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**CHANGES IN POLYMER, SCAFFOLD, AND IGF-I
DELIVERY METHODS DIRECTLY AFFECT
CARTILAGE TISSUE DEVELOPMENT**

A Dissertation Presented

By

Nichole Renee Mercier

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

June 22, 2004

CHANGES IN POLYMER, SCAFFOLD, AND IGF-I DELIVERY METHODS DIRECTLY IMPACT CARTILAGE TISSUE DEVELOPMENT

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ABSTRACT

As cartilage tissue has limited repair capacities, tissue engineering has emerged as a promising alternative for cartilage repair. The scaffold is a primary component of the tissue engineering design, yet little information exists regarding the effects of polymer and scaffold properties on tissue growth. In this study, we have developed a novel scaffold, PLG microspheres, for use in cartilage tissue engineering, which has the capacity for alterations in polymer and scaffold. We examined the effects of molecular weight, hydrophobic capping, delivery of $\text{Mg}(\text{OH})_2$, microsphere size, and controlled release of IGF-I. Our findings demonstrated that polymer parameters distinctively affect tissue and matrix output. Specifically, microspheres with high molecular weight polymer produced tissue with high GAG content and tissue mass in vivo and in vitro, while microspheres with capped polymer induced steady tissue and matrix accumulation, but may have precluded cell attachment. Release of buffer to the growing cartilage had negative effects on tissue formation in vivo and in vitro. Additionally, increasing microsphere diameter generated more samples with center of necrotic tissue. The presence of microspheres induced greater cartilage mass and matrix content than cartilage from cells alone. Delivery of IGF-I induced a dose-dependent effect on matrix and tissue production in vivo, with the highest effective load of IGF-I (0.3%) generating the most matrix and tissue accumulation. In contrast, the in vitro IGF-I dose-dependent effect induced on matrix and tissue production peaked at a dose of 0.02% IGF-I, with higher doses generating less tissue and matrix. Taken together, changes in polymer or scaffold composition and release of growth factor can be optimized to form cartilage with enhanced tissue parameters. Moreover, these results demonstrate a novel scaffold with potential to support cartilage regeneration and provide simultaneous drug delivery.

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CHAPTER I

INTRODUCTION

Cartilage Biology and Homeostasis

Hyaline cartilage is one of four cartilage classes found in the body, and articular cartilage is the type of hyaline cartilage found in synovial joints (Figure 1A). Articular cartilage is unique from other tissues in the body in that it lacks vascularity, nerves, and lymphatic vessels. Cartilage tissue is further distinguished by its ability to exist in low oxygen tensions,¹ and thus cellular energy requirements must be fulfilled by alternative methods. At its surface, cartilage tissue fulfills these energy needs by diffusion of nutrients from synovial fluid; while deeper in the tissue the chondrocytes use glycolysis to generate energy.² These metabolic processes enable chondrocytes, the functional cell of cartilage tissue, to synthesize and maintain the extracellular matrix (ECM) of articular cartilage.

The ECM (Figure 1B, C), making up only 20-40% of the total tissue wet weight³, consists of a network of collagen, proteoglycan, and non-collagenous proteins, molecules that provide the major tissue functions of resistance to mechanical loading, stress distribution and joint lubrication. The collagen molecules comprise 50-80% of cartilage dry weight,⁴ and although multiple types of collagen molecules are found in the ECM, type II collagen accounts for almost 90%.⁵ Collagen fibrils of varying types form

collagenous networks that can contact the cell via surface receptors such as integrins, and these interactions of cells with the ECM are necessary for tissue organization and maintenance since disruption of collagen-cell contacts can induce apoptosis.⁶ Although collagen networks manifest throughout the tissue, organization of collagen molecules varies with depth (Figure 1B). At the articulating surface, collagen molecules are oriented parallel to the surface, enabling stress to be distributed throughout the tissue and protecting the tissue against tensile and shear stresses. In the middle and deep zones of the tissue, collagen molecules respectively orient randomly and perpendicularly, and serve to entrap and cluster aggregating proteoglycan molecules.²

Proteoglycan molecules consist of a core protein and multiple covalently bound, sulfated glycosaminoglycan (GAG) moieties and comprise 25-35% of cartilage dry weight.² The sulfated GAGs give proteoglycans a high negative charge that hydrates the tissue, and subsequently, the high water content brings cationic charge balance.² Thus, the negatively charged proteoglycan molecules entrapped within a collagen network provide for resistance to compressive strains on the tissue.

Proteoglycans can be divided into two main categories - large aggregating proteoglycans and small proteoglycans. Aggrecan is a large aggregating proteoglycan that is considered to be a hallmark for cartilage tissue. It consists of a core protein with multiple chondroitin-sulfate and keratan-sulfate side chains. Aggrecan binds to hyaluronan in the ECM via link protein, and this tripartite complex can pack densely between collagen molecules or interact with the chondrocyte indirectly by binding to CD44 or hyaluronan (HA) synthase on the cell surface.

Unlike collagen whose half-life is extensive, aggrecan and other proteoglycans have high turnover,⁷ and this turnover of ECM is regulated by chondrocytes. In mature cartilage, chondrocytes actively engage in matrix remodeling, and although remodeling remains poorly understood, responses to mechanical loading, injury or disease are some events that induce matrix remodeling.⁸⁻¹⁰ Chondrocytes respond to these signals for remodeling by synthesizing catabolic enzymes in response to cytokines, such as interleukin-I (IL-1), that degrade matrix molecules, or making stimulatory cytokines, such as insulin-like growth factor-I (IGF-I) and transforming growth factor- β (TGF- β), that signal for chondrocytes to produce and rebuild the ECM.

This seemingly simplified matrix remodeling of catabolic and anabolic activities is a response to complex communication within the tissue. As chondrocytes make up only 2-10% of the tissue, dependent on organism, and do not maintain cell-cell interaction, signals from the ECM or cytokines are sent through receptors on the chondrocyte surface. For instance, chondrocytes have been shown to have numerous integrin clusters on the cells surface, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{11}\beta_1$, which bind to collagen type II and VI.¹¹ These integrins form focal adhesions with matrix molecules of the tissue and these focal adhesion complexes can also be a prerequisite for chondrocyte responsiveness to growth factors¹², such as IGF-I.¹⁶⁷ Other surface receptors, including annexin V, anchorin CII or CD44, also form contacts with ECM molecules, such as collagen type II or HA. Some of these interactions have been shown to modulate matrix homeostasis, and the importance of these interactions can be crucial, as blocking of CD-44 and HA binding can lead to matrix degradation.⁷

Additionally, cartilage structure and function can lead to severe limitations in endogenous cartilage repair. Due to limitations of blood flow and molecule diffusion through a dense ECM, lesions or defects caused by trauma or severe damage from diseases such as osteoarthritis cannot easily be repaired by endogenous pathways. Further, the limited repair that occasionally occurs does not integrate well with existing cartilage tissue. Thus, alternative methods to facilitate cartilage healing have been actively explored. Most recently, tissue engineering has become a promising option for cartilage repair.

Cartilage Tissue Engineering and Repair

Because tissue engineering may provide an alternative method for cartilage repair, numerous strategies have been evaluated for cartilage regeneration, including approaches using only chondrocytes, seeding cells on polymer scaffolds, and delivering growth factors to the system.

Cartilage Regeneration with Chondrocytes

In its simplest form, neo-tissue generation can occur using only chondrocytes, but chondrocyte dedifferentiation presents one caveat to cartilage tissue formation by only cells. When chondrocytes are grown in monolayer cultures in vitro they tend to dedifferentiate, and in fact, chondrocytes have been shown to lose their type II collagen expression and instead synthesize type I collagen, a marker for dedifferentiation and

fibrous-like tissue.^{13,14} Similarly, the normal spheroidal shape of the chondrocyte is lost to a more spindle-shaped cell.^{14,15}

The dedifferentiation events of monolayer culture can be circumvented by other types of in vitro culture. Chondrocytes can be maintained as pellets in suspension culture. Using these culture conditions chondrocytes have been shown to deposit a matrix of collagen type II and typical cartilage proteoglycans.^{16,17} In addition, chondrocytes retain their three-dimensional rounded morphology. In addition, the development of other 3-dimensional culture conditions have been used to maintain chondrocyte phenotype and matrix molecule production. Specifically, the use of materials such as alginate¹⁸ or agarose¹³ can be used to maintain cell populations. Recently, a method of using chondrocytes cultured in alginate beads, referred to as alginate-recovered chondrocyte (ARC), has been used to engineer cartilage.¹⁹ In the ARC culture system, chondrocytes are cultured in alginate to re-establish the proteoglycan-rich cell associated matrix, and after this matrix restoration, the cells have been seeded onto tissue culture inserts and have been shown to form cartilaginous tissue.¹⁹ Unfortunately one drawback to these cell only techniques is the hypercellularity and lack of appropriate cell spacing associated with the tissue.^{17,19}

Use of Polymer Scaffolds in Cartilage Tissue Engineering

Because of the drawbacks of cell culture methods, much of tissue engineering has focused on the use of a scaffold. As an integral dimension of the design, scaffolds have ranged from polymers that can be shaped to those that can be injected or molded. A

shaped scaffold, such as fibers²⁰ and sponges,²¹ provide a pre-defined volume and shape that the growing tissue can take. These applications have been used with various polymers, but polylactic acid (PLA), polyglycolic acid (PGA), or their copolymers (PLG) (Figure 2) have desirable characteristics such as biocompatibility and biodegradability, making them widely used to fabricate scaffolds for various tissue engineering applications. Typically, as ECM is formed and deposited onto the scaffold, the polymer degrades, and this degradation and matrix deposition has been correlated to many systems using mathematical models based on product inhibition feedback.²² Another beneficial facet of using PLG type scaffolds is the ease of polymer manipulation; properties such as polymer degradation or cell attachment can be easily modified by changes in polymer chemistry or scaffold production.²³

Unfortunately, the drawback to such shaped scaffolds is the invasive procedure of implantation, as these scaffolds must be surgically inserted in the recipient. A less invasive scaffold has been found in polymers that can be injected, such as fibrin,²⁴ photopolymerizing gels²⁵ or hydrogels.²⁶ In some cases, these types of scaffolds have been directly injected into the wound site and have been shown to mediate repair,²⁷ but these scaffolds could also be molded into specific shapes in vitro,²⁶ which can then be surgically implanted in vivo.²⁸ While the appeal of these scaffolds is their versatile use and non-invasive nature, these scaffolds cannot be readily manipulated to change characteristics such as degradation rate.

Thus, because no category of polymer scaffolds for tissue engineering fit all desirable criteria, many continue to be extensively manipulated and studied for optimal

usage. Exploration of polymer scaffolds for tissue engineering has been demonstrated both in vitro and in vivo. Evaluation in the in vitro environment is amenable to external controls with ease of repetition of culture conditions, as a known amount of growth factor, stress, and strain can be consistently delivered and monitored. Similarly, the effects of these conditions are easier to assess, since the environment is controlled and not coupled to endogenous host factors. As such, polymer scaffolds have been seeded with chondrocytes and growth factors^{24,25,29-31} or subjected to mechanical loading³¹⁻³⁵ and incubated in vitro, exhibiting varying levels of tissue growth, matrix molecules and stimulation of cell proliferation.

Although in vitro culture allows for the determination of specific effects by particular constituents, it is important that tissue engineering be assessed in vivo. Commonly, initial evaluations of novel tissue engineered designs are determined by injections or surgical implantations of the tissue engineered components into the subcutaneous pocket on the dorsal side of athymic mice.^{28,36-38} This enables assessment of the material biocompatibility without cell source limitations in autologous cell transplants. In addition to tissue evaluation of subcutaneous growth, cartilage defects have been filled with polymer and cells, using various polymer types in multiple large animal models, such as horse, sheep, and rabbit.^{25,27,39,40} Moreover, investigators have evaluated both in vitro and in vivo production of engineered cartilage, as constructs grown in vitro have been transplanted in vivo for further incubation.^{37,38}

Growth Factor Delivery and Cartilage Repair

PLG Microspheres

Another strategy for cartilage repair is to deliver proteins that can control cartilage or chondrocyte behavior. As PLG has proven biocompatibility and ease of manipulation of polymer scaffold characteristics,²³ it has been actively studied for potential cartilage tissue repair for over 2 decades. Recently, PLG has also been researched for utilization in drug delivery since the small, spherical nature of these polymers enables the encapsulation of growth factors or other drugs with subsequent controlled delivery to a specific and designated area.⁴¹⁻⁴⁵

As a drug delivery vehicle, PLG has most often taken the form of a microsphere. The feasibility of drug delivery has been demonstrated with numerous growth factors, including IGF-I, nerve growth factor (NGF), vascular endothelial growth factor (VEGF), TGF- β , and growth hormone (GH).^{25,41,42,45,46} Many of these studies have been demonstrated in vitro, but delivery of GH from PLG microspheres has also been shown in vivo. Controlled delivery of GH in vivo has been shown to elicit systemic effects of increased growth factor in circulation as well as the upregulation of other factors such as insulin-like growth factor binding protein-3 (IGFBP-3) or IGF-I.^{42,43}

Methods and patterns of controlled release have also been determined in vitro. PLG microspheres display a typical release pattern of initial bulk release followed by controlled release over an extended time period.^{41,46-48} Diffusion of growth factor tethered to the surface of the microsphere accounts for the initial bulk release from PLG microspheres, while degradation of the polymer accounts for release of protein over a

prolonged duration. Degradation of poly (α -hydroxy) acid polymers, such as PLG, occurs initially by hydrolytic cleavage of ester bonds. Once the initial hydrolytic events have begun, an autocatalytic breakdown of the polymer ensues, which is referred to as bulk hydrolysis.⁴⁹ Thus, as the polymer degrades, increasing molecules of growth factor are released to the outside environment.

PLG degradation is known to cause potential harm to growing tissue.²² Consequently, the affects of both polymer and microsphere composition must be assessed to determine whether this scaffold supports cartilage tissue regeneration, and evaluation of such can be determined by various tissue parameters including matrix accumulation, cell proliferation and overall tissue expansion. It is know that factors such as composition and geometry can affect the degradation rate of the microspheres,²³ and further that polymer degradation rate affects the composition of tissue engineered cartilage,²² possibly by mechanisms involving regulation of pH.⁵⁰ Therefore, since microspheres degrade through bulk hydrolysis of the polymer chains,⁴⁹ factors that alter microsphere degradation patterns would also be expected to affect the generation of tissue engineered cartilage. Examples of such factors include polymer molecular weight and end group chemistry. Microspheres fabricated with higher molecular weight PLG have been shown to degrade more slowly than those comprised of lower molecular weight PLG. In addition, microspheres with carboxylic acid end groups (uncapped) exhibited increased degradation rates when compared to those comprised of PLG with ester linkages (capped).²³ Due to the hydrophobic nature of the capped microspheres, water is not readily taken into the system, thereby increasing the amount of time it takes for the polymer to

degrade. Degradation rate can also be controlled through neutralizing the acidic degradation products released by the microspheres, as the encapsulation of salts and buffers has proven to neutralize the acidic environment around microspheres.²³ Thus, the influence of these parameters on polymer degradation and the surrounding environment suggests that these factors may also influence the growth of tissue engineered cartilage.

The feasibility of chondrocyte attachment to small synthetic beads and the use of PLG microspheres in cartilage tissue engineering designs have both been demonstrated independently. Microcarrier beads, which were originally used to expand anchorage-dependent cells in a suspension cell culture,⁵¹ support chondrocyte attachment and maintain the chondrocyte phenotype.⁵² In addition, PLG microspheres, as a sintered microsphere network, has provided a scaffold for osteoblast attachment and proliferation for use in bone repair.^{53,54} More recently, PLG microspheres have been combined in a cartilage tissue engineering design as controlled delivery vehicles for IGF-I or TGF- β .²⁵ Although these methods describe the culture and attachment of cells to microcarrier beads and microsphere networks or delivery of microspheres for use in cartilage tissue engineering, the use of PLG microspheres in tissue engineering has not been extensively studied and prior studies have not demonstrated delivery of chondrocytes solely via microspheres and the use of individual microspheres as a polymer scaffold for cartilage tissue engineering.

IGF-I Delivery

In tissue engineering, growth factors play an important role in stimulation of cartilage repair. Various growth factors including IGF-I has been have been shown to be active in cartilage tissue. IGF-I is a 7.6 kDa protein that signals through the IGF-I receptor on the chondrocyte membrane. Although this factor has an involvement in maintenance of cartilage homeostasis,^{55,56} it is highly regulated by the IGFBP family of proteins. IGFBPs have been shown to both inhibit and facilitate IGF-I stimulation.⁵⁷

In cartilage, IGF-I has direct effects on chondrocyte metabolism. In monolayer chondrocyte culture, cartilage explant culture, or three-dimensional seeding of chondrocytes onto polymer scaffolds, IGF-I upregulates proteoglycan synthesis and accumulation.^{24,55,56,58-61} Under similar conditions, IGF-I stimulates collagen type II on both the mRNA and protein level.^{24,60} Both collagen and proteoglycan stimulation of IGF-I are thought to be dose dependent, and typical doses used for matrix induction fall between 10 ng/ml and 300 ng/ml.^{32,61} Interestingly, however, conflicting reports have indicated differing effects of IGF-I on cell proliferation. Although IGF-I is generally considered to stimulation cell proliferation,^{24,68} some tissue engineering studies have reported that IGF-I does not increase DNA content or cell number.⁶²

IGF-I effects on matrix stimulation have also been coupled to protection against matrix breakdown. Under normal culture conditions with catabolic stimulators such as interleukin-1 (IL-1) or tumor necrosis factor (TNF), proteoglycan and collagen synthesis is suppressed and degradation of proteoglycans is evident.⁶³⁻⁶⁶ However, when cartilage explants are culture with IGF-I, proteoglycans are synthesized and the presence of IL-1 or

TNF does not induce catabolic breakdown of the tissue.⁵⁸ Thus, given the pivotal role of IGF-I in matrix synthesis and prevention of catabolic breakdown, IGF-I has been widely explored for use in cartilage tissue regeneration.

In tissue engineering applications, supplementation with IGF-I has demonstrated pronounced effects *in vitro*^{24,25,67} and *in vivo*.^{24,68} Delivery of growth factors to the developing cartilage tissue has been demonstrated through a variety of methods. *In vitro*, growth factors are commonly added exogenously, yet these methods are cumbersome and protein must be added frequently.²⁴ *In vivo* however, exogenous delivery of growth factor presents difficulties, and as a result, development of alternative growth factor release methods is actively being explored. For example, transfection of chondrocytes with expression vectors encoding growth factor genes has exhibited promising results as an alternative method for growth factor delivery. Using this system, transfected chondrocytes have been seeded in polymer scaffolds⁶⁹ or transplanted onto cartilage disks.⁷⁰ Similarly, delivery of growth factor by controlled release from a polymer scaffold has also been investigated. Gel polymers such as fibrin or alginate can release free protein^{71,72} or encapsulate a delivery vehicle such as growth factor bound heparin-sepharose beads;⁷³ both methods demonstrated sustained release over an extended period of time.

Biodegradable polymers, such as PLG, have been used for controlled release, and the feasibility of delivering IGF-I via PLG microspheres has been documented.^{25,45} Importantly, IGF-I can be stably loaded into PLG microspheres; under *in vitro* conditions, microspheres incubated in media exhibited IGF-I detection out to 2 weeks.⁷⁴

PLG microspheres loaded with IGF-I have also been used for controlled drug delivery in vivo. Rats and mice injected with a bolus dosage of IGF-I demonstrated increases in IGF-I serum levels over 2 weeks.⁴⁵ Recently, PLG microspheres were used as a growth factor delivery vehicle in tissue engineering. PLG microspheres with encapsulated IGF-I or TGF- β were included with chondrocytes in photopolymerizing gels. The effects of growth factor release in this system demonstrated increases in tissue and matrix production out to 2 weeks.²⁵

Thus, the combined promise of controlled delivery with the potential tissue engineering applications of PLG microspheres suggest that microspheres could be used as a simultaneous repair scaffold and growth factor delivery vehicle. Further, we predict that this method of IGF-I delivery from its concomitant scaffold will stimulate and sustain cartilage formation as evaluated by overall tissue production, matrix accumulation, and cell proliferation.

Hypothesis and Aims

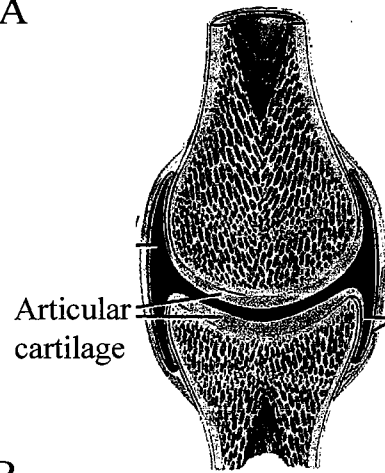
Based on previous studies documenting cell and growth factor delivery using PLG microspheres, it was the focus of this work to determine their application to cartilage tissue engineering. Therefore, we hypothesize that the presence of PLG microspheres will enhance cartilage tissue formation and that the process of chondrogenesis will be affected by variations in physical and chemical properties of the

microspheres. In addition, we predict that IGF-I delivered by controlled release will enhance chondrogenesis.

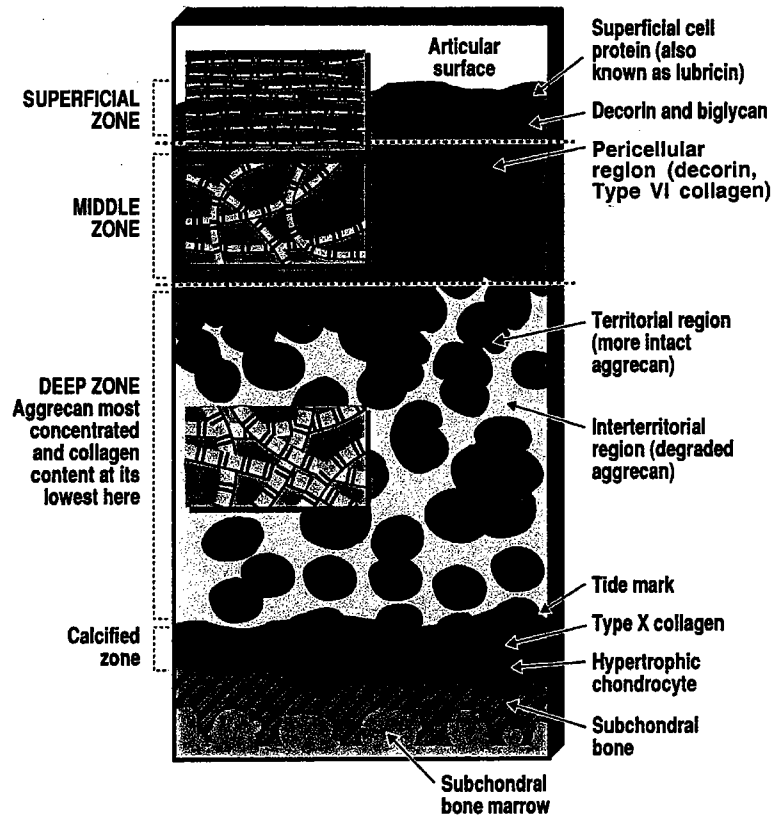
The work presented in this thesis describes two major aims investigated both in vivo and in vitro. The first aim of this work was to demonstrate that polymer and microsphere properties would affect cartilage tissue formation. The comparison of in vivo (chapter 2) and in vitro (chapter 3) studies allows for an evaluation of the contribution of the material-host interaction and systemic clearance of implant materials on the processes observed. Specifically, changes in polymer and microsphere parameters, such as factors that would affect degradation rate or surface area, could be made so as to enhance tissue and matrix production as well as cell proliferation. The interactions of cells and polymer/microsphere were evaluated both in vivo and in vitro.

The second aim of this research was to demonstrate that manipulation of the microsphere through addition of IGF-I would affect cartilage tissue formation. The inclusion of both in vivo (chapter 4) and in vitro (chapter 5) models provides for an understanding of material biocompatibility and physiological transport processes that affect cartilage tissue engineering. Specifically, the controlled release of IGF-I protein from the microsphere would act on the chondrocytes to enhance the engineered tissue. The interactions of IGF-I as released over a sustained delivery period was evaluated both in vivo and in vitro.

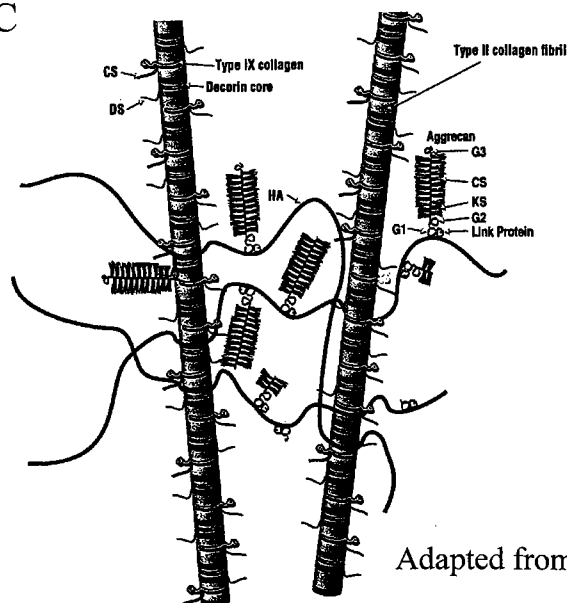
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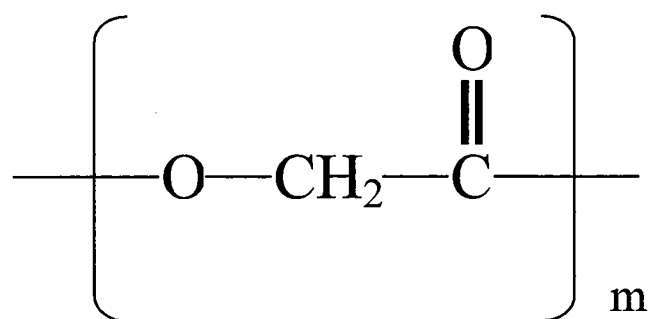
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Adapted from Poole et al. 2001

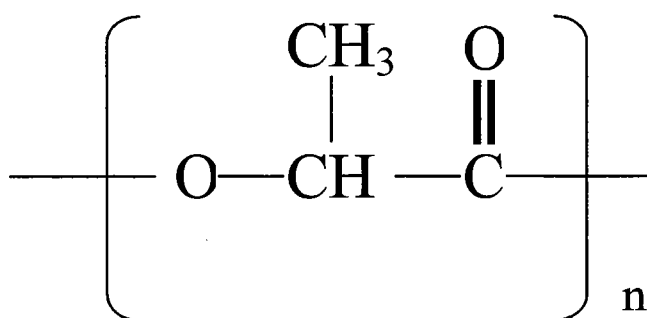
Figure 1. Articular Cartilage is located in the synovial joint (A), organized into a stratified tissue (B). Cartilage is composed mainly of collagen type II and aggrecan, which together with other matrix molecules give rise to the mechanical stability of cartilage tissue (C).

A



Polyglycolic Acid

B



Polylactic Acid

Figure 2. The structure of polyglycolic acid (A) and polylactic acid (B) differ by a single methyl group, which lends a hydrophobic characteristic to polylactic acid. PLG microspheres in this study were fabricated using a random co-polymer of polyglycolic acid residues (m) and polylactic acid residues (n), where the polymer chain was 50% polyglycolic acid and 50% polylactic acid.

CHAPTER II

A NOVEL INJECTABLE APPROACH FOR CARTILAGE FORMATION IN VIVO USING PLG MICROSPHERES⁸²

Summary

This study documents the use of biodegradable poly(lactide-co-glycolide) (PLG) microspheres as a novel, injectable scaffold for cartilage tissue engineering in vivo. Further, we demonstrate that the presence of polymer enhances tissue growth and polymer properties have distinct affects on tissue formation. Chondrocytes were delivered via injection to the subcutaneous space of athymic mice in the presence and absence of PLG microspheres. Tissue formation was evaluated up to 8 weeks post-injection. Progressive cartilage formation was observed in samples containing microspheres. The presence of microspheres increased the quantity of tissue formed, the amount of glycosaminoglycan that accumulated and the uniformity of type II collagen deposition. Microsphere composition influenced the growth of the tissue engineered cartilage. Microspheres with 50 kDa PLG resulted in a larger mass of cartilage formed and a higher content of proteoglycans. Microspheres comprised of PLG with methyl

ester caps yielded increased tissue mass and matrix accumulation, but did not display homogenous matrix deposition. The microencapsulation of $\text{Mg}(\text{OH})_2$ had negative effects on tissue mass and matrix accumulation. Matrix accumulation, cell number and tissue mass were unchanged by microsphere size, but larger microspheres increased the frequency of central necrosis in implants. Overall, we show that cartilage formed with 50 kDa PLG microspheres generated the most native-like cartilage tissue in this system and that the data reflect the promising utility of an injectable PLG-chondrocyte system for tissue engineering applications with applications for controlling tissue growth through changes in polymer properties.

Introduction

Poly(lactic acid (PLA), poly(glycolic acid (PGA), or their copolymers (PLG) are commonly used in cartilage tissue engineering. Often these polymers are constructed as fibers²⁰ or sponges,²¹ which can be seeded with cells and implanted into the recipient. One drawback to these scaffolds, however, is the invasive procedure of implantation: these fibers and sponges must be surgically implanted into the recipient. Poly(lactide co-glycolide) (PLG) microspheres offer a non-invasive alternative to the aforementioned polymer designs.

PLG microspheres made of varying polymer types^{42,75} have predominately been used as controlled drug delivery vehicles, and their biocompatibility and injectability are appealing for cartilage tissue engineering applications. Furthermore, the small, spherical

nature of these polymers enables the encapsulation of growth factors or other drugs and their subsequent delivery to a specific and designated area.

The feasibility of chondrocyte attachment to small synthetic beads and the use of PLG microspheres in cartilage tissue engineering designs have both been demonstrated independently. Microcarrier beads, which were originally used to expand anchorage-dependent cells in a suspension cell culture,⁵¹ have been demonstrated as a cell carrier system. Cells have been shown to attach to microcarrier beads made of dextran, collagen type I coated dextran, or highly cross-linked collagen type I and that culture on these microcarriers maintains the chondrocyte phenotype.⁵² More recently, PLG microspheres have been used in a cartilage tissue engineering design as controlled release delivery vehicles for IGF-I or TGF- β .²⁵ Although these methods describe the culture of chondrocytes on microbead carriers or delivery of microspheres in the use of cartilage tissue engineering, neither has demonstrated delivery of chondrocytes solely via microcarrier/microsphere for cartilage tissue engineering.

Fibrin glue or hydrogels such as agarose or alginate have also been used for cartilage tissue engineering.^{26,27} While these are attractive scaffolds for their injectability, they do not possess the encapsulation capabilities that PLG microspheres offer. As such, growth factor delivery to the cartilage tissue construct must be added exogenously to the scaffold-cell construct.³¹ Further, these scaffolds do not have properties that readily enable changes in the degradation rate, whereas PLG polymer chains can be manipulated to change the scaffold properties.²³

Factors such as composition and geometry are known to affect the degradation rate of the microspheres.²³ Polymer degradation rate is known to affect the composition of tissue engineered cartilage,²² possibly by mechanisms involving regulation of pH.⁵⁰ Since microspheres degrade through bulk hydrolysis of the polymer chains,⁴⁹ factors that alter microsphere degradation patterns would also be expected to affect the generation of tissue engineered cartilage. These factors include polymer molecular weight and end group chemistry. For instance, microspheres made from higher molecular weight PLG degrade more slowly than those comprised of lower molecular weight PLG. Yet another example is the increased degradation rate for microspheres with carboxylic acid end groups (uncapped) compared to those comprised of PLG with ester linkages (capped).²³ Due to the hydrophobic nature of the capped microspheres, water is not readily taken into the system, thereby increasing the amount of time it takes for the polymer to degrade. Degradation rate can also be controlled through neutralizing the acidic degradation products released by the microspheres. The encapsulation of salts and buffers has proven to neutralize the acidic environment around microspheres.²³

The influence of these parameters on polymer degradation and the surrounding environment suggests that these factors may also influence the growth of tissue engineered cartilage. The goal of this study was to examine the feasibility of using PLG microspheres as a delivery vehicle for chondrocytes. Using techniques to determine GAG production, tissue mass, collagen type, and tissue morphology the effects of microsphere size, buffer encapsulation, polymer molecular weight, and polymer end group chemistry on the production of tissue engineered cartilage was assessed.

Materials and Methods

Cell Culture

Chondrocytes were isolated from the articular cartilage of calf gleno-humeral joints of 2-10 day old calves (Research 87, Hopkinton, MA) using 0.3% type II collagenase (Worthington), as previously described.⁷⁶ After 16 hr of collagenase treatment at 37°C, isolated chondrocytes were filtered through a 180 µm filter to remove any undigested cartilage particles, washed several times with PBS, and cells were resuspended in Ham's F12 media supplemented with 1% ascorbic acid and 10% FBS.

Microsphere preparation

PLG was obtained from Alkermes, Inc. PLG microspheres were fabricated using the ProLease[®] process, as described.²³ Briefly, 50:50 lactide:glycolide polymer was dissolved in methylene chloride and atomized over a bed of liquid nitrogen layered on top of frozen ethanol. The temperature of the system was then maintained at -80°C to allow for nitrogen evaporation and removal of methylene chloride into the ethanol phase (microsphere curing). The resulting microspheres were filtered and then lyophilized to remove residual ethanol, and finally sieved to obtain the desired range of particle sizes. PLG microspheres were prepared in groups with average diameter ranging from 63-199 µm. Microspheres were also formed using polymers with varying molecular weight and

capped end groups chemistries (Table 1). Separate batches of microspheres were prepared with $\text{Mg}(\text{OH})_2$ salt. The salt was homogenized in the solution of polymer and methylene chloride, and the suspension was processed as described above.

Culture Conditions and Implantation

Chondrocytes, at a density of 80×10^6 cells/mL, were mixed with equal volume of PLG microspheres, at a concentration of 64 mg/mL. The microsphere-chondrocyte suspension was incubated for 4 hr at 37°C in a shaker incubator to allow for chondrocyte attachment to the microspheres.

Chondrocyte, microsphere, and chondrocyte-microsphere suspensions were injected into the subcutaneous space of the dorsum of nude mice using an 18-gauge needle. Each injection contained 20×10^6 cells and/or 16 mg of microspheres in 500 μL of F12 media ($n=3-6$ per group tested).

Sample Analysis

All animal procedures were performed under the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. Mice were sacrificed at 1, 2, 4, 6, and 8 weeks by anesthesia overdose and samples were harvested from cell alone and chondrocyte-microsphere samples. No tissue samples could be harvested from microsphere alone injections, as there was no tissue formation at the site of injection.

Upon harvest, the fibrous capsule was removed from each sample. The sample was weighed and cut in half. Half of the sample was digested with 0.14 mg papain (Sigma Chemical Co., St. Louis, MO) for 16 hr at 60°C. Papain digested samples were subjected to biochemical analysis for cell number and proteoglycan content. The other half of the sample was fixed in 10% formalin buffer. The fixed sample was embedded in paraffin and sectioned. Sections were stained with safranin-O. Unstained sections were probed for collagen type I and type II by immunohistochemistry as described below.

Immunohistochemistry

Detection of collagen types I and II was achieved by immunohistochemical staining as described by Madry et al.⁷⁰ Briefly, unstained paraffin sections were deparaffinized, rehydrated and washed in PBS with 0.1% BSA. Samples were incubated with 0.3% hydrogen peroxide, washed, and subjected to digestion for 30 min with 33U/mL bovine testicular hyaluronidase (Sigma Chemical Co., St. Louis, MO). Sections were incubated with a 1:50 dilution of anti-collagen type I (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) for 48 hr at 4°C or a 1:10 dilution of anti-collagen type II (Chemicon, Temecula, CA) for 2 hr at room temperature, washed, and incubated with a 1:200 dilution of anti-IgG biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Sections were washed and subjected to avidin-biotin-peroxidase reagent (Vector Laboratories, Burlingame, CA), washed, and exposed to diaminobenzidine (Vector Laboratories, Burlingame, CA) for 2 min. Sections were counterstained with 1% alcian blue for 3 min for collagen type I staining or 0.02% fast

green for 4 min for collagen type II staining. Digital photomicrographs were obtained using a Nikon TE 200 microscope equipped with a Spot Junior camera (both from Micro Video Instruments, Avon, MA).

DNA Quantification

Papain digested samples were added to an equal volume of phenol-chloroform-isoamyl alcohol (Sigma Chemical Co., St. Louis, MO). Sample tubes were inverted several times and then centrifuged in a tabletop microcentrifuge at 12,000 x g for 5 min. The aqueous supernatant was removed from the protein fraction to a clean tube and 1 mL of cold 100% ethanol was added to the aqueous layer. Samples were stored at -20°C overnight to precipitate DNA. DNA was pelleted by centrifugation at 12,000 x g for 30 min, and upon removal of ethanol, DNA was dried overnight and resuspended in dH₂O. DNA content was read by spectrophotometry at 260 nm and the purity was determined by the 260/280 ratio.

Proteoglycan Content Analysis

Papain digested samples were analyzed for glycosaminoglycan (GAG) content using the DMMB-dye binding assay.⁷⁷ Briefly, 50 µL of papain digested sample was incubated with 2 mL of DMMB-dye and the reaction was observed on a spectrophotometer at 525 nm, with shark chondroitin sulfate (Sigma Chemical Co., St. Louis, MO) used as a standard.

Statistical Analysis

All statistics were performed using Sigma StatTM Statistical Software Version 2.0. To detect statistical differences between tissue mass, proteoglycan accumulation or DNA content, two-way ANOVA tests were performed with post-hoc Bonferroni test.

Results

At all time points, samples were recovered from sites that were injected with cells alone or cells and microspheres. No samples were recovered from sites where microspheres alone were injected. Histological analyses of the retrieved cartilage samples demonstrated the presence of microspheres interspersed with newly forming cartilage after 1 week (Figure 3A). Chondrocytes remained rounded and those adjacent to the microspheres appeared to be attached to the polymer (Figure 3A). After 2 weeks post-injection, microsphere degradation was evident and newly synthesized matrix was deposited in the region of the polymer (Figure 3B). Cell attachment to microspheres was also still apparent. After 4 weeks, some fragments of PLG microspheres remained and there was some evidence of cell attachment to the remaining material (Figure 3C). Extracellular matrix was uniformly deposited across the entire specimen including into areas formerly occupied by microspheres. In contrast, cartilage generated from cells alone contained fibrous tissue interspersed between cartilage regions (Figure 3D). Moreover, the cartilaginous regions of the cell-only tissue were hypercellular in comparison with cartilage from cells and polymer, which exhibited lower cell density and larger distances between cells.

Given that the presence of PLG microspheres appeared to enhance cartilage formation, subsequent studies focused on the effects of microsphere composition on the growth of tissue engineered cartilage. Parameters studied included molecular weight, polymer end group, microsphere contents, and microsphere size.

Histological analysis of these samples showed that after two weeks matrix had been formed in all samples (Figure 4). However, there were distinct trends in matrix deposition between the different samples. Cartilage grown from cells alone exhibited many areas of fibrous looking tissue and areas with an intense number of cells (Figure 4C). In all cases, cartilage matrix was not deposited in the area of the injection site until the microspheres began to degrade. Specifically, in samples harvested from fast degrading, uncapped, low molecular weight microspheres, matrix deposition appeared to be uniform as early as two weeks (Figure 4A), whereas in slower degrading microsphere samples, such those containing high molecular weight or capped microspheres, the proteoglycan matrix was interrupted by undegraded microspheres that left empty gaps of staining (Figure 4B,E). At later time points uniform deposition was observed in fast degrading microsphere samples (Figure 4D), and as the slower degrading microspheres began bioeroding, matrix accumulated in the injection site area (Figure 4F). After 8 weeks, matrix remained distinct from microsphere areas in cartilage formed using capped microspheres (Figure 4E).

Tissue mass steadily increased over 8 weeks of culture in samples formed from 50 kDa microspheres and capped microspheres (Figure 5A). Growth from 10 kDa, uncapped microspheres increased slightly until 6 weeks, but more than doubled after 8

weeks of culture. In contrast, samples formed with buffered microspheres initially exhibited high tissue mass, but as early as 6 weeks drastically decreased in mass. Tissue mass of samples formed chondrocytes cartilage alone remained unchanged up to 6 weeks, after which there was a sharp increase in tissue mass.

Upon harvest of samples after two weeks, cell number is slightly increased from the initial 20×10^6 cell seeding density (Figure 5B). However, in all samples seeded with microspheres, there was a steady decline in cell number until 6 weeks, when cell density reached steady state at half of the initial seeding density. Cell number did not vary with polymer composition or in samples without microspheres. Cell number dropped more quickly in samples with chondrocytes alone, decreasing to less than half the initial number at 4 weeks.

GAG content remained steady in samples made from low molecular weight, uncapped, and unbuffered microspheres, while samples made from capped microspheres, high molecular weight microspheres or cells alone showed increased GAG content over 8 weeks of culture (Figure 5C). In contrast, cartilage formed with buffered microspheres exhibited decreased matrix content after 6 weeks. Moreover, cartilage grown using 50 kDa microspheres had significantly higher proteoglycan content than buffered microspheres after 8 weeks ($p < 0.02$). As well trends showed that cartilage tissue grown using 50 kDa microspheres had higher proteoglycan content than cartilage formed using 10 kDa, capped, or cells alone.

Tissue engineered cartilage samples were immunostained for type I and type II collagen (Figure 6). Cartilage grown with microspheres and chondrocytes exhibited

positive staining for type II collagen (Figure 6D) and negative staining for type I collagen (Figure 6C). In contrast, however, cartilage formed from chondrocytes alone exhibited many pockets of fibrous looking tissue that stained positive for collagen type I expression (Figure 6A).

Samples formed from different sized microspheres exhibited similar matrix deposition patterns over 8 weeks of culture (Figure 7). After two weeks (Figure 7A,B) matrix was not apparent on microspheres of any size, while after 8 weeks, the degradation of all microspheres encouraged matrix deposition across the tissue specimen including microsphere regions (Figure 7C,D). However, after two weeks tissue areas surrounding the larger microspheres stained less intensely for proteoglycans (Figure 7B).

Microsphere size did not appear to affect mass of generated tissue. Use of microspheres ranging in size from 59 μm to 199 μm resulting in a steady increased in tissue mass over 8 weeks post-injection, but there were no significant differences at any time point (Figure 8A). However, these studies further emphasize the difference in tissue generation following injection of the microsphere-chondrocyte system compared to administration of chondrocytes alone, where mass remained relatively unchanged over 8 weeks.

Furthermore, microsphere size affected total cell number (Figure 8B). Cell number was doubled from the initial seeding density after 8 weeks in samples formed from 199 μm , 121 μm , or 93 μm , which were significantly increased in cell number when compared to the 59 μm polymer or cartilage formed from cells alone ($p < 0.02$). Cell number was lowest in samples from cells alone.

Microsphere size appeared to have no influence on proteoglycan content.

Proteoglycan accumulation steadily increased in all cartilage samples after 8 weeks in vivo (Figure 8C). However, the only statistical differences in GAG content occurred at 6 weeks with 199 μm samples producing significantly higher GAG content than cells alone or 59 μm samples.

Some samples formed from larger microspheres exhibited a necrotic cavity when harvested. When these samples were cross sectioned and stained with safranin-O the inner cavity was hollow (Figure 9A,B). The percentage of samples exhibiting central necrosis increased with microsphere size (Figure 9).

Discussion

This study demonstrates the feasibility of using microspheres as a novel injectable cell delivery system for cartilage tissue engineering. The majority of literature on PLG microspheres to date describes their use in drug or growth factor delivery^{41,42,75}. However, recent studies have used microspheres in tissue engineering in combination with scaffolds such as PLG⁷⁸ or PEO.²⁵ We demonstrate herein that chondrocytes attach to PLG microspheres and form cartilage tissue. This type of application has broad ranging possibilities for filling skeletal defects, site specific enhancements, and non-invasive tissue engineering procedures.

In this study, we demonstrate that injectable chondrocyte delivery using PLG microspheres produced cartilage tissue that is more similar to native cartilage than that grown from cells alone, both in uniformity of tissue, matrix formation, and in cell-cell

spacing. Matrix deposition remained distinct from the microspheres prior to polymer biodegradation. Moreover, there was an associated hypercellularity before degradation of the microspheres; cells appeared packed in non-microsphere regions at the early time points. As biodegradation began, cells appeared to migrate into the spaces left by degrading microspheres and matrix was deposited on top of and in the regions of the degrading microspheres. At these later time points, cartilage formed from cells alone continued to exhibit these areas of hypercellularity. Further, many of these hypercellular areas were separated by fibrous tissue, which was comprised primarily of type I collagen. Matrix deposition patterns in conjunction with the degradation patterns of the polymer suggest that the microspheres act as a tissue bulking agent; the polymer saves spaces for the formation of new tissue and the deposition of newly synthesized matrix.

This was further evidenced by the tissue mass data. The mass of material recovered from the site of administration continued to increase up to 8 weeks post-injection. As the 8 weeks progressed, the polymer continued to degrade and newly formed chondrocyte tissue accumulated, accounting for the increase in mass. It was also evident that faster degrading microspheres resulted in a lower mass recovered 8 weeks post-injection. It is hypothesized that the higher tissue mass in the 50 kDa samples was likely a product of effective spacing for a longer time, thereby providing more areas for tissue accumulation. These observations are consistent with theoretical models of cartilage tissue formation that predict similar effects on tissue composition due to changes in scaffold degradation rate.²²

Since degradation time was also increased by capping the polymer,²³ substituting a methyl ester cap for the free carboxylic end group would seemingly increase the bulking effect of the tissue. Although we saw a gradual increase in tissue mass after 8 weeks, these tissue samples only exhibited a moderate increase in GAG content. Moreover, histological analysis showed that the distribution of matrix in capped polymer samples remained distinct from microsphere regions up to 8 weeks. It has been suggested that polymers with increased hydrophobicity, such as polylactic acid,⁷⁹ do not promote cell attachment or proliferation as well as hydrophilic polymers. Thus, although the microspheres provide a moderate bulk effect to the growing tissue, a lack of cell adhesion to the hydrophobic, capped microspheres may explain the continued presence of microspheres and small amount of matrix deposition onto microspheres after 8 weeks.

The rate of degradation and its effects on tissue synthesis are related to the rate of release of acidic degradation fragments. Degradation in poly (α -hydroxy) acid polymers occurs initially by hydrolytic cleavage of ester bonds. Once the initial hydrolytic events have begun, an autocatalytic breakdown of the polymer ensues, which is referred to as bulk hydrolysis.⁴⁹ During the phase of bulk hydrolysis, the local pH of the polymer environment may drop due to the inability of these acidic end fragments to diffuse efficiently. Drops in local pH have been demonstrated to inhibit cartilage matrix assembly.⁸⁰ Other studies have noted that maintaining a stable pH leads to increased matrix production.⁵⁰

In an attempt to buffer these acid fragments, $\text{Mg}(\text{OH})_2$ salt was encapsulated in the microspheres. This method has been shown to maintain local pH²³ around degrading

microspheres and would be expected to provide a more favorable environment that would promote cell proliferation and increase matrix production. Contrary to expectations, the cartilage formed with buffered microspheres yielded less tissue with lower mass and GAG content as early as 6 weeks, although cell number was not different from other microsphere samples. It is possible that excessive $\text{Mg}(\text{OH})_2$ release may have increased the local pH, which has been shown to have negative effects on cell metabolism.⁵⁰

Changing microsphere size impacts the microsphere surface-to-volume ratio and available area for cell attachment. Histological analysis of safranin-O stained sections indicated less staining around larger microspheres, suggesting local effects of increased degradation fragments. Although samples with increased microsphere size exhibited higher cell number, no corresponding increase in tissue mass or proteoglycan accumulation was observed. One possible rationale for this observation could be the higher number of samples made from larger microspheres exhibiting central necrosis.

The issue of central necrosis is thought to be a limiting factor in the generation of large volumes of engineered tissues. This phenomenon is thought to be related to a lack of transport of oxygen to central regions of the growing tissue.⁸¹ The findings (figure 9) from the current study indicated that the composition of the polymer scaffold also regulates the process of central necrosis. Low molecular weight microspheres, which generate acidic degradation fragments at a faster rate, and larger microspheres, which potentially localize greater numbers of degradation fragments, both were seen to be associated with higher rates of central necrosis. It is important to note that the size of all samples were similar, such that diffusion distances, and thus oxygen transport, were

similar in all samples. Together, this suggests that, in this system, clearance of degradation fragments, and not oxygen transport, appears to be the primary factor regulating central necrosis.

Overall, samples formed using cells and microspheres exhibited between 14-36 μg of GAG per mg tissue and 0.3-1.9 μg DNA per mg tissue after 8 weeks, which is similar to values found for engineered cartilage tissue.^{26,37} However, cartilage formed from high molecular weight microspheres produced the optimal tissue in this study (Table 2). These samples produce the highest amount of GAG and total tissue mass without exhibiting central necrosis of the sample.

In summary, the results presented herein demonstrate the potential utility of an injectable PLG microsphere-chondrocyte system for tissue engineering applications. The effects of polymer chemistry and microsphere size were elucidated; at optimal conditions, rapid formation of native-like cartilage occurred compared to delivery of cells alone. It is anticipated that the same approach will have potential applications in engineering of other important tissues types.

Acknowledgements

The authors would like to acknowledge Phong Dargon for his help with immunostaining.

Polymer	Size (um)	M _W (kDa)	End Group	Deg. Rate (week)	Buffer
2A	52	10	COOH	2-4	-
2A	58	10	COOH	2-4	+
2M	68	10	COOCH ₃	6-8	-
4A	59	50	COOH	6-8	-
4A	93	50	COOH	6-8	-
4A	121	50	COOH	6-8	-
4A	199	50	COOH	6-8	-

Table 1. Microsphere composition variables. Microspheres differed with respect to size, molecular weight, end group, degradation rate, and buffer additions. Microspheres described as low molecular weight are composed of 10 kDa polymer, while 50 kDa is considered high molecular weight. Capped microspheres have polymer chains ending with COOCH_3 , while uncapped microspheres polymer chains end with COOH . A plus symbol indicates the addition of $\text{Mg}(\text{OH})_2$ in the microspheres (see methods).

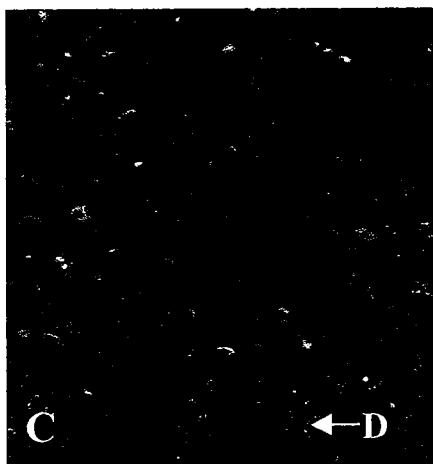
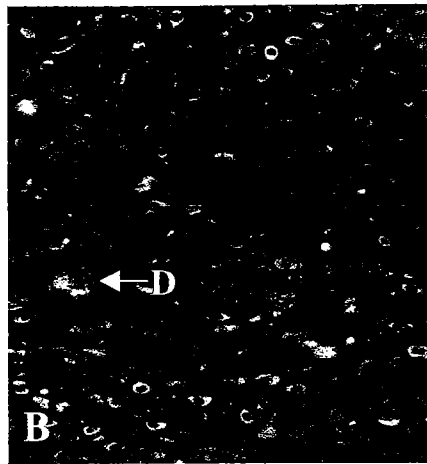


Figure 3. Cartilage matrix replaces degraded microspheres. Chondrocytes and microspheres (A-C) or chondrocytes alone (D) were incubated for 4 hours and injected subcutaneously in athymic mice for 1 (A), 2 (B) or 4 weeks (C,D). All microsphere samples used low molecular weight, uncapped microspheres. Intact microspheres are indicated by S; Degrading microspheres are indicated by D; yellow arrowheads indicate areas of possible dedifferentiation. Note that microspheres may appear to have a range of sizes due to a difference in the plane of sectioning. All pictures were magnified at 200x.

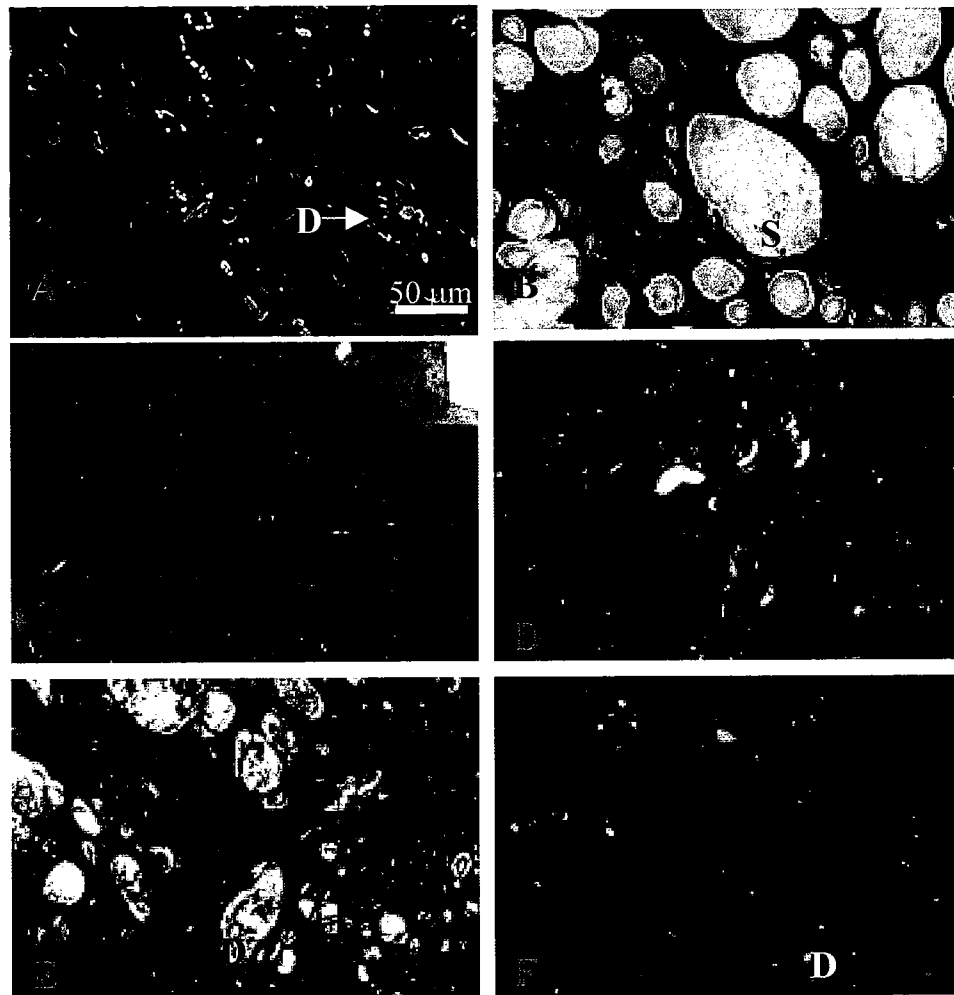


Figure 4. Cartilage tissue matrix deposits differently with respect to polymer composition. Tissue sections were embedded in paraffin and sectioned. Samples were stained with safranin-O for proteoglycans. A. Fast degrading, uncapped microspheres, 2 weeks; B. Slow degrading, capped microspheres, 2 weeks; C. Cells alone, no microspheres, 8 weeks; D. Fast degrading, uncapped microspheres, 8 weeks; E. Slow degrading, capped microspheres, 8 weeks; F. Slow degrading, uncapped microspheres, 8 weeks. All pictures were taken at 200x magnification. Black arrowheads indicate areas of possible dedifferentiation.

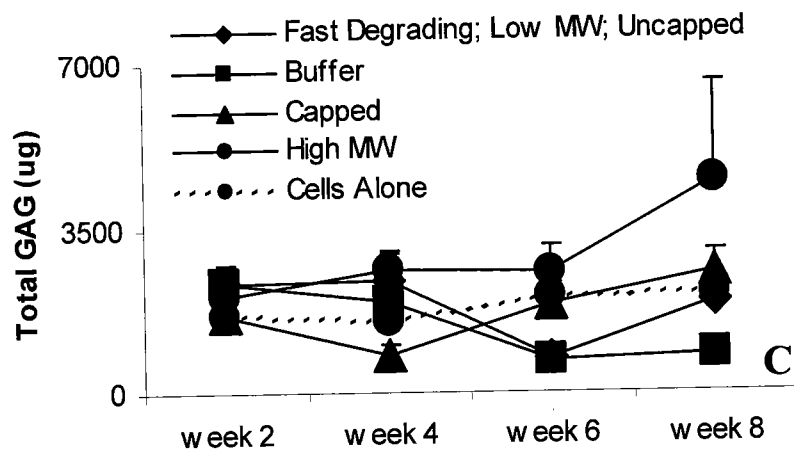
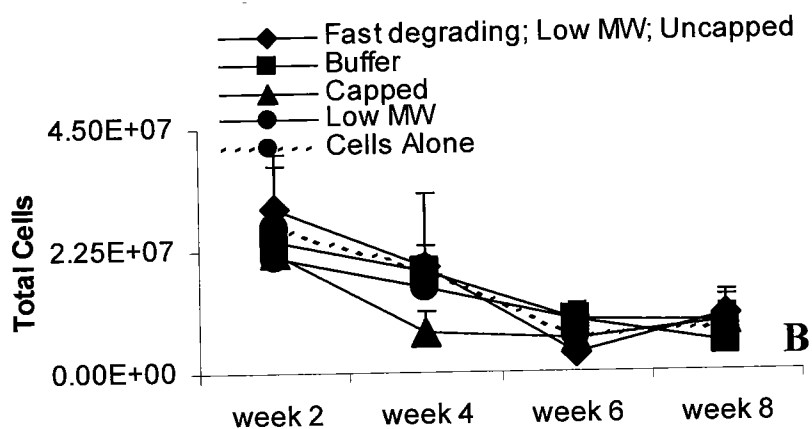
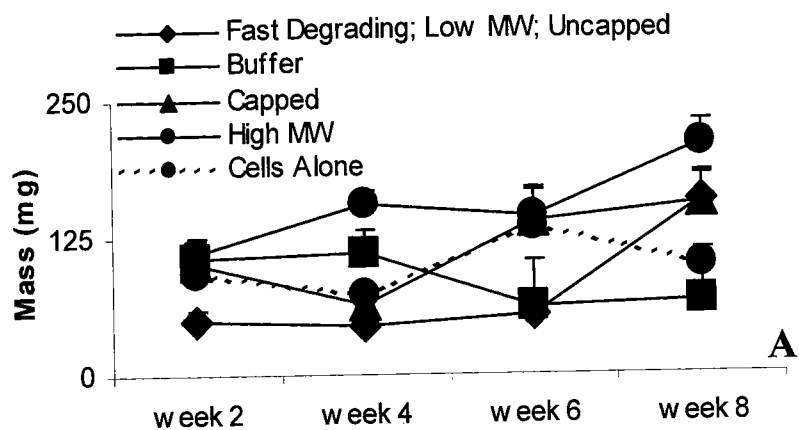


Figure 5. Biochemical analysis of polymer composition on cartilage tissue formation. A. Tissue mass varies with polymer composition. Samples were harvested, fibrous capsule was removed, and wet weight of the samples was taken. Samples formed with higher molecular weight microspheres exhibited higher tissue mass than other groups, although these differences were not statistically significant. B. Cell content did not vary between groups. DNA was extracted from papain digested samples using phenol-chloroform and DNA content was determined by spectrophotometer readings at 260 nm. C. GAG content varies with polymer composition. Biochemical samples were digested with papain and proteoglycan content was determined using the DMMB-dye method. Samples formed using high molecular weight microspheres were higher in GAG content than other groups, but were only statistically higher in GAG content than buffered microspheres after 8 weeks ($p < 0.02$). $n=3$ for buffered microsphere samples, $n=4$ for capped microsphere and uncapped, 10 kDa microsphere groups, and $n=6$ for 50 kDa microsphere samples.

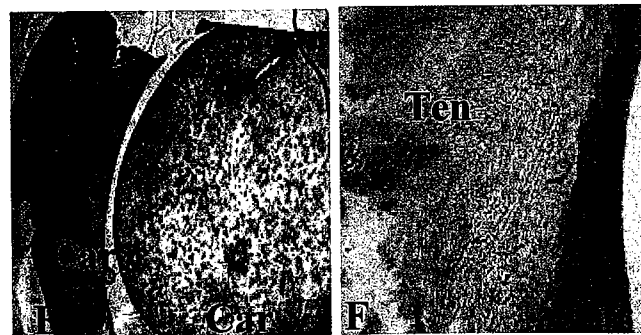
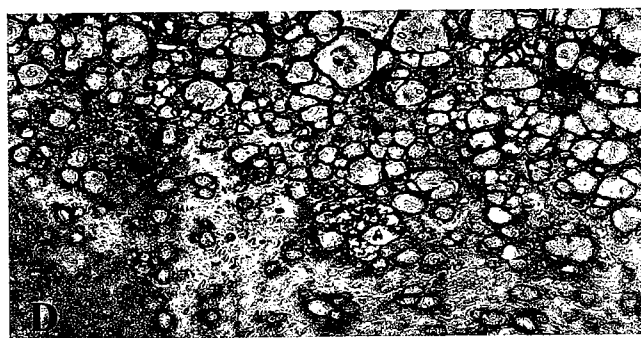
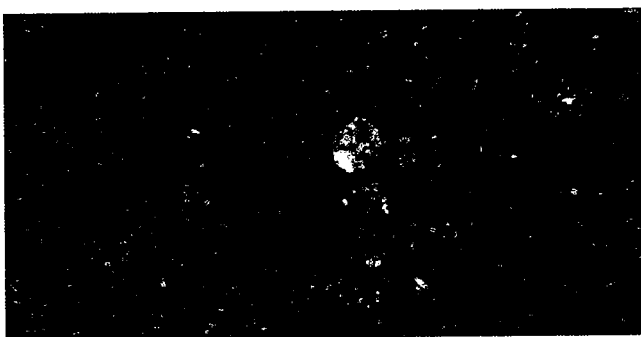
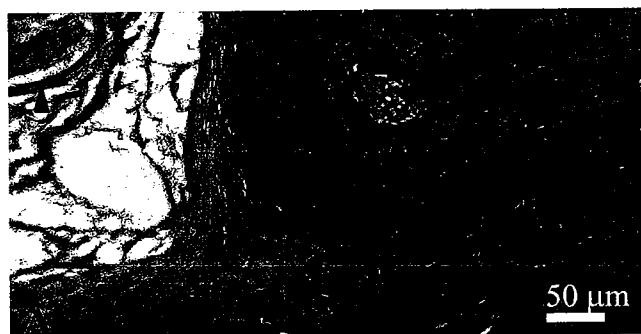


Figure 6. Immunostained sections for collagen types. Cartilage tissue made from cells alone (a,c) or cells and microspheres (b,d) was embedded in paraffin and sectioned. Sections were immunostained as described for collagen type I (a,b) or collagen type II (c,d). Control cartilage (Car) and tendon (Ten) were also immunostained for collagen type II (e) and type I (f). Positive staining for either collagen type I (black arrowhead) or collagen type II is indicated by brown. Negative staining for collagen type I is indicated by blue. A green color indicates negative staining for collagen type I (red arrowhead). All pictures were magnified at 100x.

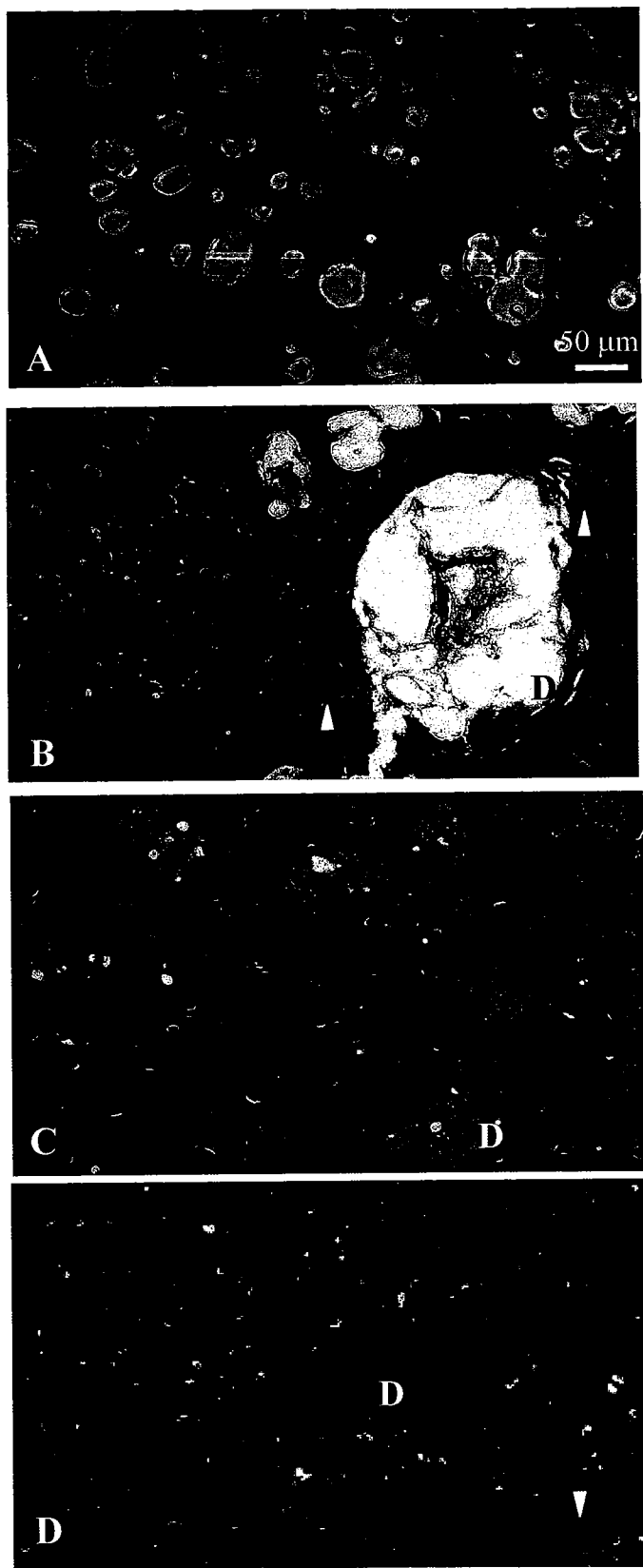


Figure 7. Cartilage tissue matrix deposition in samples from different sized microspheres. Tissue sections were stained for safranin-O in 59 μm samples (A,C) or 199 μm samples (B,D). Matrix was distinct from microspheres after two weeks (A,B), but homogenous for proteoglycans after 8 weeks (C,D). Microsphere size does not affect tissue mass. Samples were harvested, fibrous capsule was removed, and samples were weighed. Black arrowheads indicate areas of light staining around large microspheres. All pictures are 200x magnification. n=4 for all samples

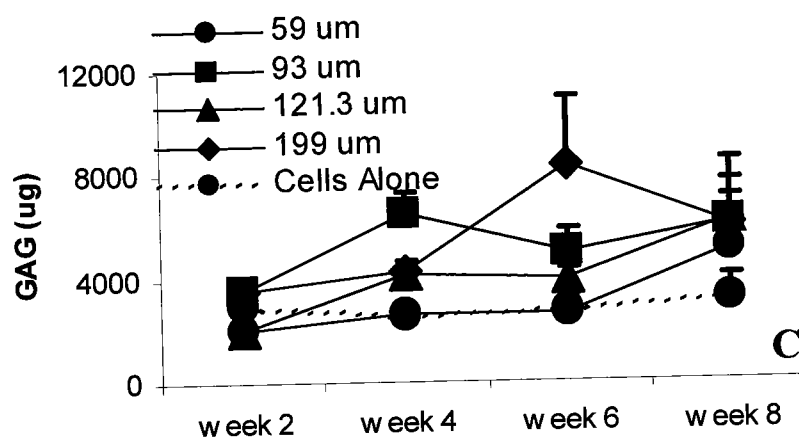
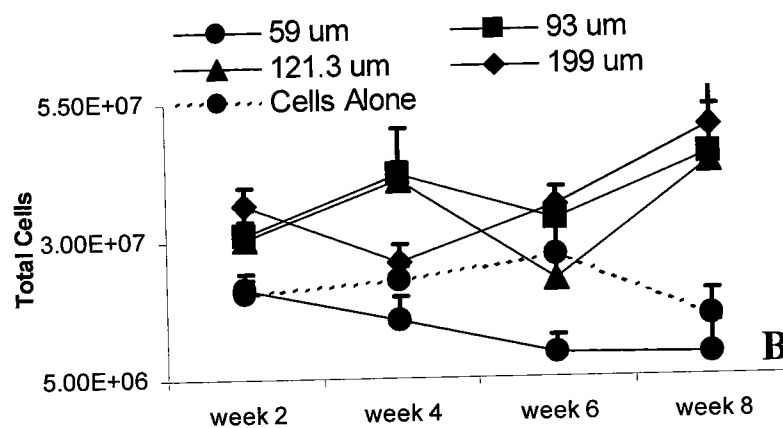
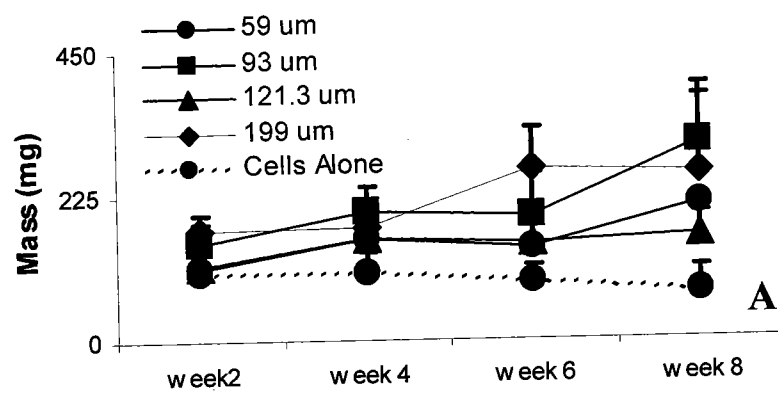


Figure 8. Biochemical analysis of the effects of microsphere size on cartilage tissue formation. A. Tissue mass does not vary with microsphere size, but samples formed from PLG microspheres are significantly higher than cartilage formed from cells alone ($p < 0.006$). B. Cell content is significantly higher in samples formed from larger microspheres (93-199 μm) than from 59 μm or cells alone ($p < 0.02$). C. GAG content does not vary with microsphere size. $n=4$ for all groups.

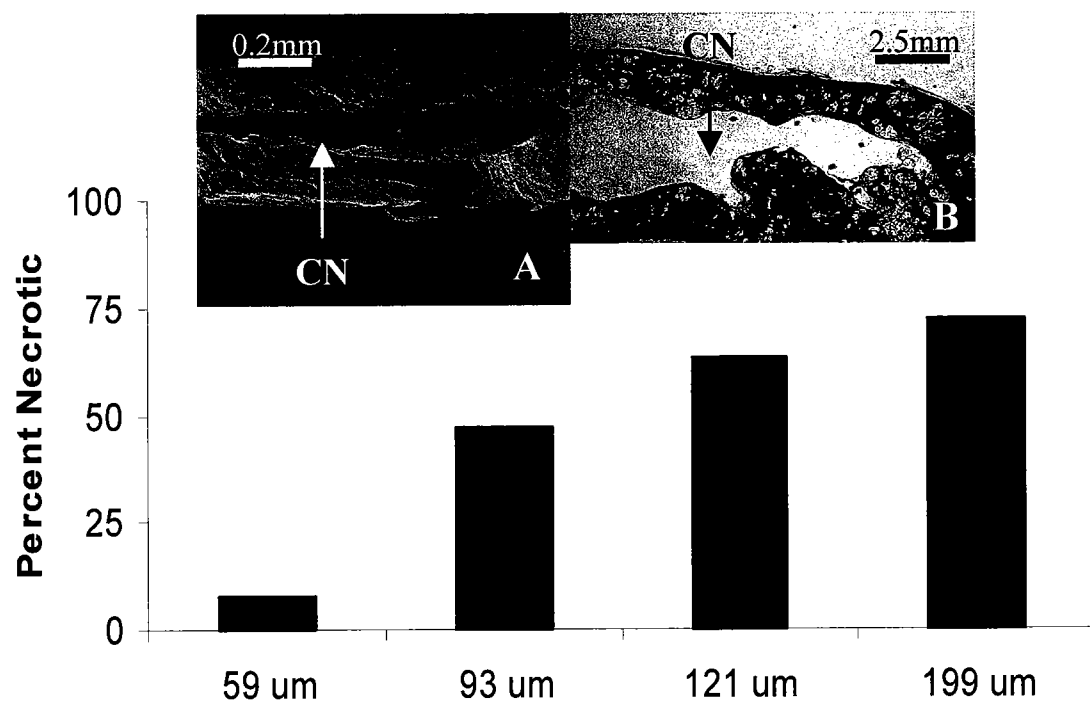


Figure 9. Central necrosis increases with microsphere size. A. Bulk frozen fracture scanning electron micrograph of central cavity. B. Cross section of safranin-O stained sample exhibiting central necrosis. C. Percentage of samples exhibiting central necrosis. Necrotic areas are indicated by CN designation. n=4 for all groups.

Variable Tested	Overall Outcome	Associated Figure
Low Molecular Weight, Uncapped	No real increase in tissue or GAG accumulation from 2-8 w weeks	Figure 5a, 5b
Buffering	Has negative affects on tissue mass and GAG production	Figure 4d, 5a, 5b
Capping	Increased total tissue, but not GAG production	Figure 5a, 5b
High Molecular Weight	Increased tissue and GAG production	Figure 5a, 5b

Table 2. Summary of overall results. Cartilage harvested from the different conditions suggest that cartilage formed from high molecular weight microspheres enhance GAG production and tissue mass, while not exhibiting central necrosis.

CHAPTER III

PLG MICROSPHERES AS A MOLDABLE SCAFFOLD FOR CARTILAGE TISSUE ENGINEERING¹⁶⁶

Summary

This study documents the use of biodegradable poly(lactide-co-glycolide) (PLG) microspheres in vitro as a moldable scaffold for cartilage tissue engineering and evaluates the effects of microsphere composition on tissue growth in vitro. First, distinct differences were noted between cartilage generated with only cells and that grown with microspheres and chondrocytes. Specifically, cartilaginous tissue formed using chondrocytes and microspheres maintained thickness, shape, and chondrocyte phenotype, as indicated by type II collagen production. The presence of microspheres further enhanced total tissue mass and the amount of glycosaminoglycan that accumulated. Second, microsphere properties, such as polymer molecular weight, end group chemistry, and buffer inclusion, have direct and different effects on tissue growth. Specifically, higher molecular weight PLG resulted in a larger mass of cartilage-like tissue formed and a higher content of proteoglycans. Cartilage-like tissue formed using microspheres made from low molecular weight and free carboxylic acid end groups did not display increases in tissue mass, yet modest increased proteoglycan accumulation was detected. Microspheres comprised of PLG with methyl ester end groups yielded a steady increase

in tissue mass, with no real increase in matrix accumulation. Samples formed with microencapsulated $\text{Mg}(\text{OH})_2$ indicated decreased tissue mass and matrix accumulation. The results gained from this study were consistent with those results from the in vivo cartilage regeneration model. Moreover, the data herein reflect the potential utility of a moldable PLG-chondrocyte system for tissue engineering applications and demonstrate that scaffold properties can be changed to control tissue growth.

Introduction

Many different types of polymers have been used for cartilage tissue engineering. Some of the most common polymers include polylactic acid (PLA), polyglycolic acid (PGA), or their copolymers (PLG), often in the form of fibers²⁰ or sponges.²¹ These polymers have been known for their biocompatibility and can be easily seeded with cells and implanted into the recipient. PLG has also been used to form microspheres, whose small, spherical nature enables the encapsulation of buffers, growth factors or other proteins for controlled release.^{23,25,42} Although PLG microspheres have been predominately used for controlled drug delivery, more recently, they have been demonstrated for use in tissue engineering for drug delivery⁵ or as an injectable scaffold^{8,2}. Although PLG microspheres and chondrocytes have been delivered in vivo via direct injection,⁸² they could potentially be used in vitro as a moldable scaffold. Current polymers used as moldable scaffolds include fibrin glue or alginate,^{26,27} yet these scaffolds do not possess modulatory structural and chemical properties that could potentially optimize tissue growth.

In contrast, PLG polymer chains can be manipulated to change the scaffold properties.^{23,82} Engineered tissue growth in vivo was affected when the compositional make-up of the polymer used in the fabrication of PLG microspheres was altered.⁸² Changes that alter molecular weight to increase degradation time also increased matrix accumulation, whereas microspheres loaded with a buffering agent had negative effects on matrix accrual. Other studies suggest that polymer degradation rate can affect the composition of engineered cartilaginous tissue,²² possibly by mechanisms involving regulation of pH.⁵⁰ Since microspheres degrade through bulk hydrolysis of the polymer chains,⁴⁹ factors that alter microsphere degradation patterns would also be expected to affect the generation of tissue engineered cartilage.

As such, specific changes in microspheres composition have been demonstrated to influence degradation rate including polymer molecular weight, end group chemistry, and the controlled release of a buffering agent. For instance, microspheres made from higher molecular weight PLG degrade more slowly than those comprised of lower molecular weight PLG. Yet another example is the increased degradation rate for microspheres with carboxylic acid end groups (uncapped) compared to those comprised of PLG with ester linkages (capped).²³ Due to the hydrophobic nature of the capped microspheres, water is not readily taken into the system, thereby increasing the amount of time it takes for the polymer to degrade. Degradation rate can also be controlled through neutralizing the acidic degradation products released by the microspheres. The encapsulation of salts and buffers has proven to reduce the PLG degradation rate, likely due in part to neutralization of acidic byproducts from microsphere degradation.²³

In the current investigation, it was desired to expand on these observations and examine the feasibility of microsphere-mediated construction of cartilage-like tissue in vitro. The in vitro environment is more amenable to external controls; biochemical or mechanical conditions can be easily repeated, as a known amount of growth factor, stress, strain, etc. can be consistently delivered and monitored. Similarly, the effects of these conditions are easier to assess, since the environment is controlled and not coupled to undefined endogenous factors. In vitro culture also offers the ability to mold cells and polymer simultaneously into a shaped implant.²⁶

The influence of various parameters on polymer degradation and engineered cartilage-like tissue in vivo suggests that these factors may also influence the growth of engineered tissue in vitro. The goal of this study was to examine the feasibility of using PLG microspheres in vitro as a moldable scaffold for cartilage tissue engineering. Using techniques to determine GAG production, tissue mass, collagen type, and tissue morphology the effects of buffer encapsulation, polymer molecular weight, and polymer end group chemistry on the production of tissue engineered cartilage-like tissue were assessed.

Materials and Methods

Cell Culture

Chondrocytes were isolated from the articular cartilage of calf gleno-humeral joints of 2-10 day old calves (Research 87, Hopkinton, MA) using 0.3% type II collagenase (Worthington), as previously described.⁷⁶ After 16 hr of collagenase treatment at 37°C,

isolated chondrocytes were filtered through a 180 μm filter to remove any undigested cartilage particles, washed several times with PBS, and cells were resuspended in Ham's F12 media supplemented with 1% ascorbic acid and 10% FBS.

Microsphere preparation

PLG was obtained from Alkermes, Inc. PLG microspheres were fabricated using the ProLease[®] process, as described.²³ Briefly, 50:50 lactide:glycolide polymer was dissolved in methylene chloride and atomized over a bed of liquid nitrogen layered on top of frozen ethanol. The temperature of the system was then maintained at -80°C to allow for nitrogen evaporation and removal of methylene chloride into the ethanol phase (microsphere curing). The resulting microspheres were filtered and then lyophilized to remove residual ethanol, and finally sieved to obtain the desired range of particle sizes. PLG microspheres were prepared in groups with average diameter ranging from 52-68 μm . Microspheres were also formed using polymers with varying molecular weight and capped end groups chemistries (Table 3). Separate batches of microspheres were prepared with $\text{Mg}(\text{OH})_2$ salt. The salt was homogenized in the solution of polymer and methylene chloride, and the suspension was processed as described above.

Culture Conditions and Implantation

Chondrocytes, at a density of 80×10^6 cells/mL, were mixed with equal volume of PLG microspheres, at a concentration of 64 mg/mL. The microsphere-chondrocyte

suspension was incubated for 4 hr at 37°C in a shaker incubator to allow for chondrocyte attachment to the microspheres.⁸²

For in vitro culture, the wells of a 12-well plate were covered with a surface layer of 0.5% agarose to prevent cell adhesion to the bottom of the well, and a cylinder measuring 1 cm in diameter was placed in the well. Agarose solidification sealed the cylinder to the well. Chondrocytes alone, microspheres alone, and a mixed system of chondrocytes and microspheres were delivered as a 500 µL volume to the cylinders. Each delivery contained 20×10^6 cells and/or 16 mg of microspheres in 500 µL of F12 media (n=2-4 per group tested).

Sample Analysis

Upon harvest, each sample weight was taken, which included the mass of the microspheres, and cut in half. Half of the sample was digested with 0.14 mg papain (Sigma Chemical Co., St. Louis, MO) for 16 hr at 60°C. Papain digested samples were subjected to biochemical analysis for cell number and proteoglycan content. The other half of the sample was fixed in 10% formalin buffer. The fixed sample was embedded in paraffin and sectioned. Sections were stained with safranin-O. Unstained sections were probed for collagen type I and type II by immunohistochemistry as described below.

Immunohistochemistry

Detection of collagen types I and II was achieved by immunohistochemical staining as described.^{70,82} Briefly, unstained paraffin sections were incubated with a 1:50 dilution

of anti-collagen type I (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) for 48 h at 4°C or a 1:10 dilution of anti-collagen type II (Chemicon, Temecula, CA) for 2 h at room temperature, washed, and incubated with a 1:200 dilution of anti-IgG biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Sections were counterstained with 1% alcian blue for 3 min for collagen type I staining or 0.02% fast green for 4 min for collagen type II staining. Digital photomicrographs were obtained using a Nikon TE 200 microscope equipped with a Spot Junior camera (both from Micro Video Instruments, Avon, MA).

DNA Quantification

DNA quantification was performed as described.⁸² Briefly, papain digested samples mixed with phenol-chloroform-isoamyl alcohol (Sigma Chemical Co., St. Louis, MO). DNA was extracted and content was read by spectrophotometry at 260 nm and the purity was determined by the 260/280 ratio. A known number of chondrocytes was used as a standard for calculation of cell number.

Proteoglycan Content Analysis

Papain digested samples were analyzed for glycosaminoglycan (GAG) content using the DMMB-dye binding assay.⁷⁷ Briefly, 50 µL of papain digested sample was incubated with 2 mL of DMMB-dye and the reaction was observed on a spectrophotometer at 525 nm, with shark chondroitin sulfate (Sigma Chemical Co., St. Louis, MO) used as a standard.

Statistical Analysis

All statistics were performed using Sigma StatTM Statistical Software Version 2.0. To detect statistical differences between tissue mass, proteoglycan accumulation or DNA content, two-way ANOVA tests were performed with post-hoc Bonferroni test for pairwise comparison.

Results

Samples were harvested from wells containing cells alone or cells and microspheres. The gross morphology of harvested samples suggested major differences in sample structure. Tissue specimens harvested from cells alone formed a thin layer of tissue over the agarose layer and often picked up the coloration of the media (Figure 10A). After 8 weeks, tissue formed from cells alone took a disk-like shape, but the tissue remained translucent and thin (Figure 10B). In contrast, samples formed from cells and microspheres had a defined disk-like shape at early time points (Figure 10C) that continued to increase in tissue firmness, thickness and height with incubation time (Figure 10D).

Microsphere composition also affected the gross morphology of the tissue. Samples formed from microspheres made from low molecular weight and uncapped polymer, high molecular weight polymer, or capped polymer formed defined cartilage-like disks. However, samples formed from buffered microspheres formed tissue samples that appeared less stable when harvested (data not shown).

Samples stained with safranin-O demonstrated differences between tissue formed from cells alone or from cells and high molecular weight microspheres. Samples formed from cells alone appeared hypercellular throughout the study, but stained uniformly with safranin-O (Figure 11A, Table 3). In contrast, staining patterns clearly demonstrate the presence of microspheres, as well as exclusion of matrix deposition from these areas until the microsphere began to degrade (Figure 11B).

Tissue mass steadily increased in samples formed from cells alone or cells and high molecular weight microspheres over 8 weeks of culture. At each time point samples formed with cells and microspheres had a 3 to 4-fold increase over tissue formed from cells alone ($p < 0.001$) (Figure 12A).

Initially, GAG content was similar in samples formed from cells and microspheres and those formed with cells alone. All samples increased in GAG content throughout the 8 weeks, however, differences were seen as early as 4 weeks. Samples formed using cells and microspheres had a 5-fold difference in GAG content after 4 weeks and a 2.5-fold difference in GAG content after 8 weeks (Figure 12B).

When cell content was analyzed, a significant decrease was noted in the number of cells in samples formed from cells alone from 2 to 8 weeks ($p < 0.004$). However, the total number of cells remained fairly constant in samples formed with cells and microspheres. Total cell number was 30-50% of the initial cell load in both sample sets (Figure 12C).

Histological analysis of samples formed from cells and microspheres further revealed differences between tissues formed using microspheres of different polymer

compositions. In all samples, microspheres did not begin to degrade until after 4 weeks and at the later time points, microsphere remains were still detectable. As well, safranin-O staining did not appear in the area of the microspheres until degradation of the polymer occurred. Staining in samples formed from microspheres of 50 kDa or 10 kDa, uncapped polymer appeared uniform across the tissue both at early and late time points (Figure 13A, B, E). At early time points, tissue formed from microspheres containing buffer exhibited homogenous staining across the tissue (Figure 13C), yet as early as 6 weeks, these samples exhibited minimal staining for proteoglycans (Figure 13D). Additionally, the microspheres in samples formed using buffered microspheres were not easily observed (Figure 13D). After 6 weeks, matrix deposition in microsphere regions was apparent in samples formed using microspheres of high molecular weight polymer (Figure 13E), while samples formed using microspheres with capped polymer did not deposit matrix in the microsphere areas at late time points (Figure 13F).

Tissue mass was assessed in samples formed with different microsphere compositions. In general, samples increased in tissue mass over the 8 weeks of culture. At intermediate time points, samples formed from microspheres with buffer or high molecular weight polymer had the highest tissue mass, which was significantly higher than other polymer counterparts ($p < 0.04$) (Figure 14A). However, samples formed using buffered microspheres did not form compact and dense tissue, as was formed with all other microsphere samples. After 8 weeks samples formed with buffered microspheres had significantly decreased weight from those harvested at 4 or 6 weeks ($p < 0.03$) (Figure 14A).

Proteoglycan content also varied with polymer composition. Samples formed from microspheres with low molecular weight, uncapped or capped polymer demonstrated modest increases in GAG content, while the GAG content in samples formed from buffered microspheres remained stagnant throughout the time course (Figure 14B). In contrast, samples formed with microspheres of high molecular weight polymer had an overall increase in GAG content over the time course. After 8 weeks, GAG content had a 2.5-fold minimum difference from tissue formed with different microsphere compositions ($p < 0.005$) (Figure 14B).

At the first time point of 2 weeks, cell number was decreased in all samples to about half of the initial cells delivered. These numbers did not vary significantly throughout the 8 week time course and no differences were detected between any of the samples (Figure 14C).

Tissue samples were immunostained for collagen type I and collagen type II (Figure 15). All samples stained negative for collagen type I, as evidenced by the blue counterstain (Figure 15A, C). In contrast, the homogenous brown coloration indicated positive staining in all tissue sections for collagen type II (Figure 15B, D).

Discussion

The majority of literature on PLG microspheres to date describes their use in drug or growth factor delivery,^{41,42,75} but recent studies examine their potential in tissue engineering.^{25,53,82} PLG microspheres can be fabricated to achieve changes in the microsphere composition,^{23,82} which have been shown to affect cartilage-like tissue

growth in vivo.⁸² This study indicates that PLG microspheres can be used as a moldable scaffold in vitro, which would have use in filling specific cartilage defects or in applications where complex geometries are needed. In this study, the presence of polymer served to bulk cartilage-like tissue, allowing it to be shaped into disks that maintained thickness and shape, while preserving additional space for matrix accumulation.

Although tissue engineering often involves a polymer scaffold, several studies have investigated the utility of implants composed of cells alone. Chondrocytes have been cultured in vitro as pellets¹⁶ or in a method known as alginate recovered chondrocytes,^{19,83} as these methods maintain the chondrocyte phenotype of a well developed collagen and proteoglycan matrix. Although phenotype maintenance and retention can be argued,^{16,17} these culture systems exhibit hypercellularity and a lack of cell spacing,^{17,19} consistent with the results demonstrated in this study in cartilage-like tissue from chondrocytes alone. In contrast, chondrocytes in tissue formed with PLG microspheres appeared densely packed when microspheres were still intact, but as microspheres began to degrade, the chondrocytes appeared more appropriately spaced, consistent with in vivo observations.⁸² Similarly, tissue formed from chondrocytes and microspheres exhibited a cell density that was more similar to native cartilage tissue than chondrocytes cultured in the absence of a polymer scaffold (Table 4).

Distinctions between tissue grown with cells and microspheres or with cells alone were further evidenced by tissue analyses. Cartilage-like tissue formed using chondrocytes and microspheres maintained a significantly higher tissue mass throughout

the 8 weeks of study. In addition, after 4 weeks in culture the matrix accumulation in samples formed using microspheres remained greater than cartilage-like tissue formed from cells alone and exhibited almost 50% of matrix accumulation in native cartilage tissue (Table 2). Interestingly, although total cell number did not differ between the two groups, the amount of GAG/DNA suggested that fewer cells were responsible for larger accumulation of matrix. These differences suggest that PLG microspheres provide a scaffold for engineered cartilage-like tissue in vitro that enhances tissue and matrix growth, and this enhancement could be attributed to limitations in oxygen diffusion. The potential benefits of low oxygen tension have indicated stimulation of chondrocyte redifferentiation and increases in GAG content.^{84,85} Because the diffusional distance would be greater in samples formed with microspheres, the oxygen tension in these samples is likely lower and may promote more optimal growth conditions than methods involving cell alone delivery.

Given that PLG microspheres enhance tissue and matrix growth, it is important to determine whether microsphere composition affects engineered tissue growth in vitro. Microsphere degradation in vitro is known to be slower than degradation in vivo²³. Data from cartilaginous tissue formed with fast degrading and uncapped polymer indicated that microsphere degradation was detected as early as 2 weeks.⁸² In contrast, cartilage-like tissue formed in vitro from microspheres with the same composition displayed no evidence of degradation before 4 weeks (Figure 13). Similarly, cartilage-like tissue formed using slower degrading microspheres exhibited microsphere breakdown by 4 weeks in vivo, while in vitro microspheres with buffer, capped polymer or high

molecular weight polymer remained intact until the 6 week time point. After 4-6 weeks in vitro, matrix was deposited on microspheres made from fast degrading, uncapped polymer or high molecular weight polymer, respectively, but in microspheres formed with capped polymer matrix remained excluded and cells were densely packed in the spaces not occupied by microspheres.

Interestingly, cell number remains largely unchanged in the different samples, suggesting that matrix deposition inside the regions of the microspheres may be due to cell migration and resultant changes in cells spacing. Moreover, the hydrophobic cap may contribute to the lack of cell spacing demonstrated in vitro and in vivo,⁸² as polymers with increased hydrophobicity, such as polylactic acid,⁷⁹ do not promote cell attachment or proliferation as well as hydrophilic polymers. In addition, a lack of cell adhesion to the hydrophobic, capped microspheres may explain the continued presence of microspheres, small amount of matrix deposition onto microspheres, and minimal proteoglycan accumulation after 8 weeks.

We also tested the effects of buffer release to the growing cartilage-like tissue, since encapsulation of $\text{Mg}(\text{OH})_2$ salt has been shown to decrease microsphere degradation rate²³. Release of buffer would be expected to provide an environment that might counteract microsphere bulk hydrolytic breakdown⁷⁸ that causes decreases in pH, which in turn has been shown to mediate matrix integrity.^{50,80} However, in vitro cartilage-like tissue formed from buffered microspheres negatively affected tissue mass and matrix accumulation, consistent with in vivo data.⁸² In this study, the negative effects of the buffer were noted as early as 6 weeks by evidence of poor sample staining with safranin-

O and drastic decreases in proteoglycan accumulation and cell number, suggesting that release of buffer might be a contributing factor in the poor tissue growth.

In summary, we report for the first time a PLG microsphere-chondrocyte system suitable for in vitro production of molded cartilaginous tissue. Characteristics of cartilage-like tissue produced in vitro were similar to those of tissue produced in vivo using these microspheres,⁸² with consistent and specific effects of changes in the polymer scaffold on developing tissue. The characteristics of the cartilage-like tissue produced by this in vitro system are native-like and may have utility for tissue engineering applications of multiple tissue types.

Acknowledgements

The authors would like to thank Phong Dargon for his help with immunostaining.

Polymer	Size (um)	M _w (kDa)	End Group	Deg. Rate (week)	Buffer
Fast degrading, uncapped	52	10	COOH	2-4	-
Buffered	58	10	COOH	2-4	+
Capped	68	10	COOCH ₃	6-8	-
High molecular weight	59	50	COOH	6-8	-

Table 3. Microsphere composition variables. Microspheres differed with respect to size, molecular weight, end group, degradation rate, and buffer additions. Microspheres described as low molecular weight are composed of 10 kDa polymer, while 50 kDa is considered high molecular weight. Capped microspheres have polymer chains ending with COOCH_3 , while uncapped microspheres polymer chains end with COOH . A plus symbol indicates the addition of $\text{Mg}(\text{OH})_2$ in the microspheres (see methods).

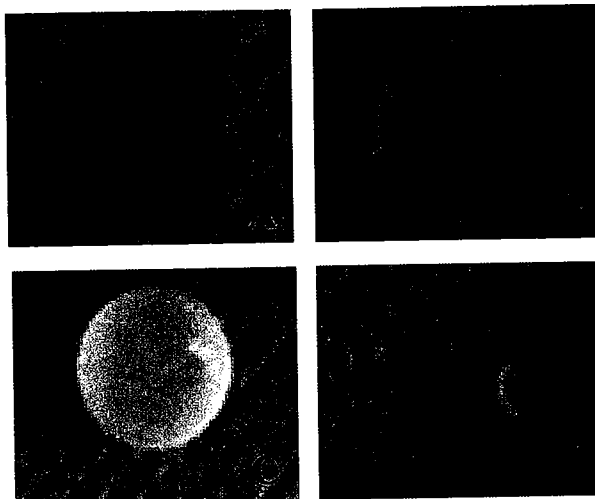


Figure 9. Gross morphology of in vitro cartilaginous disks. Cartilage disks were formed using chondrocytes alone (A, B) or chondrocytes and 50 kDa PLG microspheres (C, D). Cloning cylinders, 1 cm in diameter, were used to mold cartilage-like samples. Samples were harvested at 2 weeks (A, C) and 8 weeks (B, D). The diameter of the disk was 1 cm in B-D, whereas cartilage-like tissue from chondrocytes only at 2 weeks exhibited a 40% reduction in diameter.

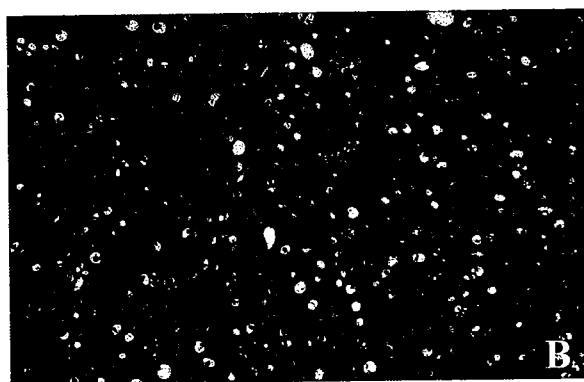
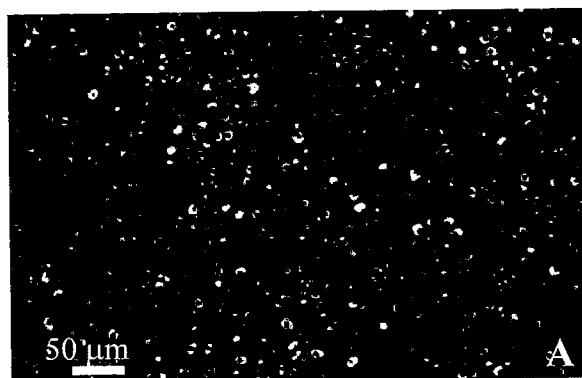


Figure 10. Histological analysis of cartilaginous disks stained with Safranin-O. A. Cartilage-like tissue formed from chondrocytes alone at 8 weeks. B. Cartilage-like tissue formed from chondrocytes and PLG microspheres at 8 weeks. Cartilage formed from cells alone exhibited hypercellularity and lack of appropriate cell spacing, while cartilage formed with cells and microspheres had more appropriate cell spacing after 8 weeks. All pictures were magnified 100x.

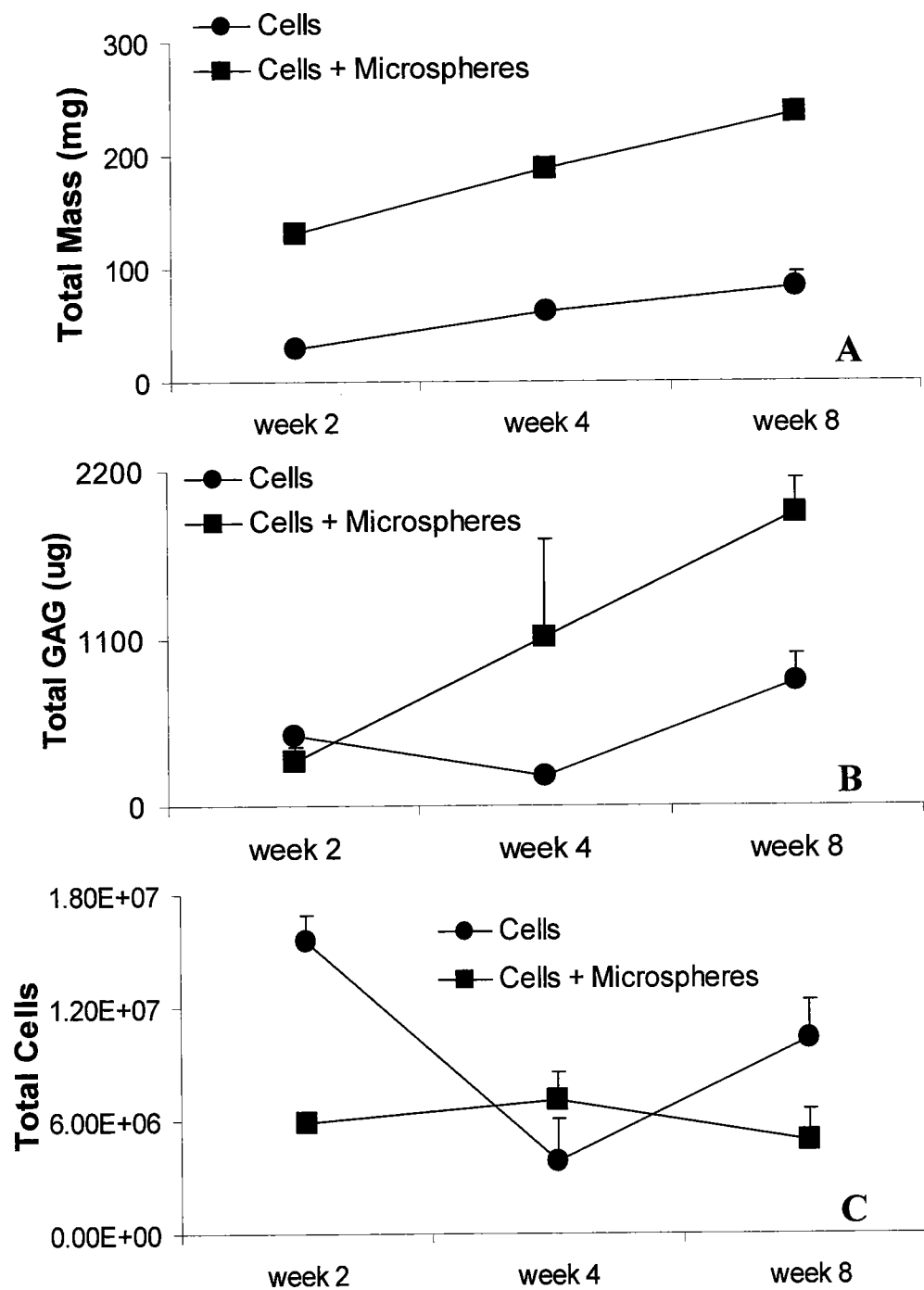


Figure 11. Biochemical analysis of in vitro cartilaginous tissue growth using PLG microspheres. A. Cartilage-like tissue formed in the presence of PLG microspheres demonstrated higher tissue mass than tissue formed from cells alone. B. Cartilage-like tissue formed with cells and microspheres exhibited increased GAG content. C. Cell content did not vary between samples made from cells and microspheres or from cells alone. $n=4$ for all groups.

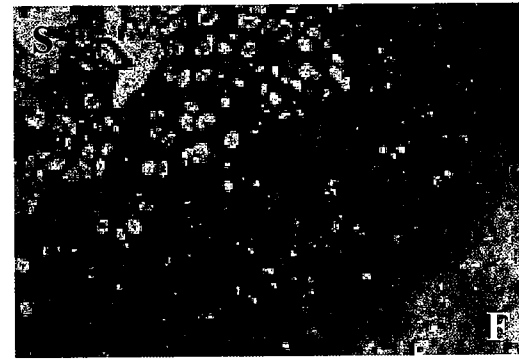
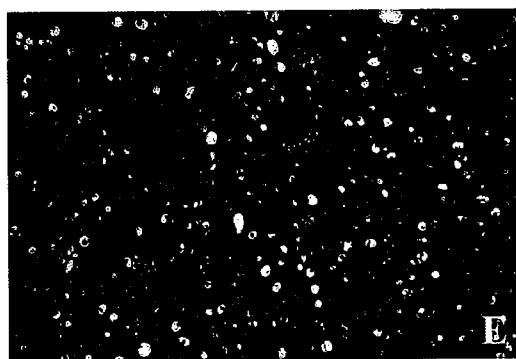
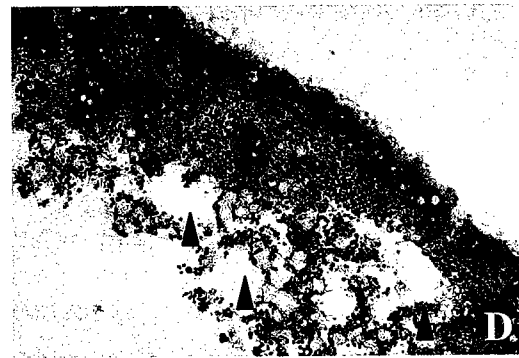
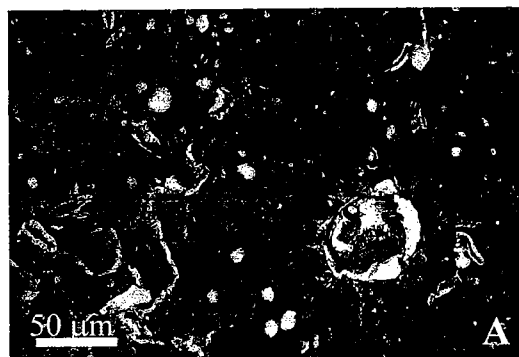


Figure 12. Histological analysis of matrix deposited in the growing cartilage-like samples. Tissue sections were cut from paraffin embedded samples and stained with safranin-O. A. Fast degrading, uncapped microspheres at 4 weeks; B. Fast degrading, uncapped microspheres at 6 weeks; C. Buffered microspheres at 4 weeks; D. Buffered microspheres at 6 weeks; E. High molecular weight microspheres at 6 weeks; D. Capped microspheres at 6 weeks. All pictures are magnified 200x. Intact microspheres and bioeroding microspheres are differentiated by (S) and (D), respectively. Black arrowheads indicate possible areas where microspheres may be present in cartilaginous tissue formed with buffered microspheres. Note that microspheres may appear to have a range of sizes due to a difference in the plane of sectioning.

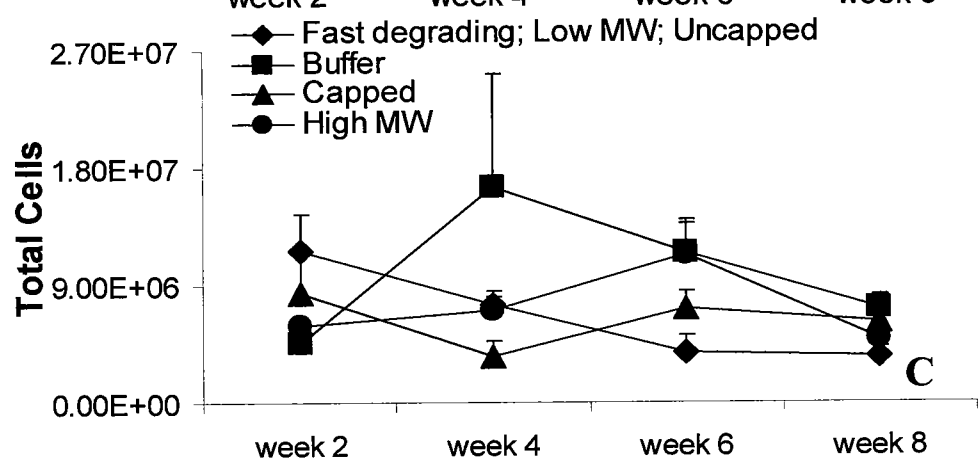
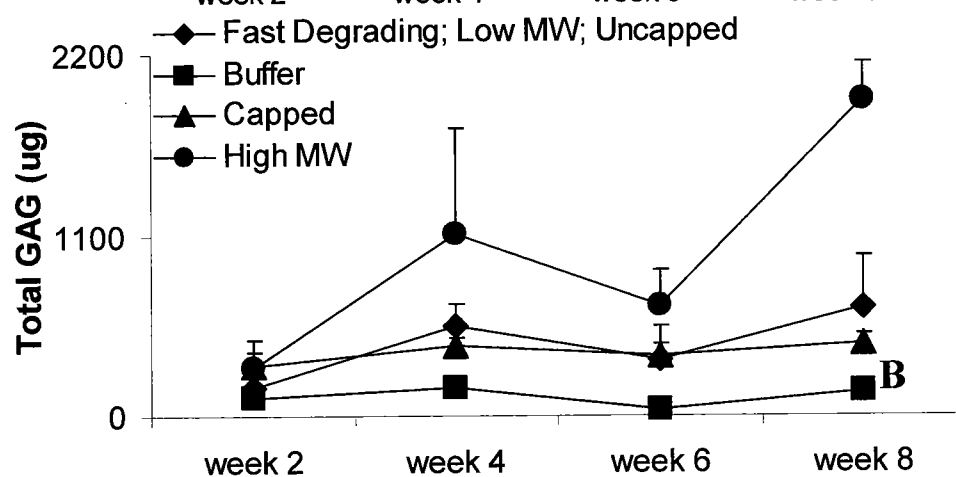
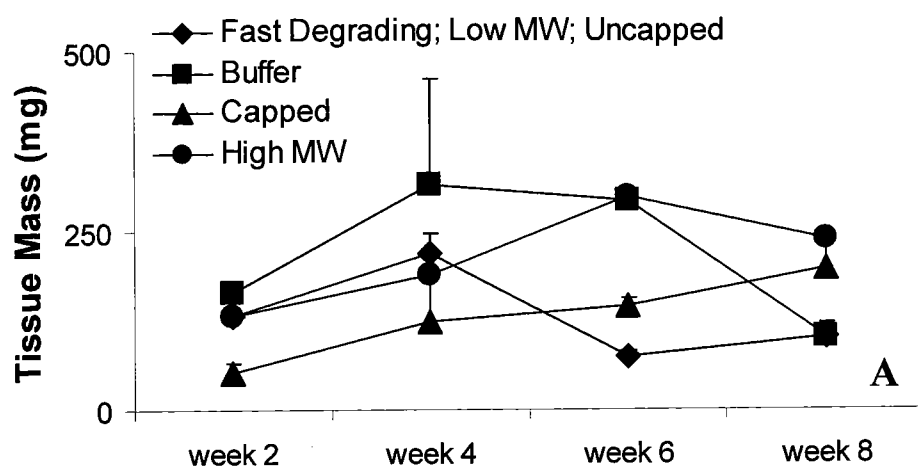


Figure 13. Biochemical analysis of polymer composition on in vitro cartilage-like tissue formation. A. Tissue mass varies with polymer composition. Samples were harvested, fibrous capsule was removed, and wet weight of the samples was taken. Samples formed with higher molecular weight microspheres exhibited higher tissue mass than other groups, although these differences were not statistically significant. B. Cell content did not vary between groups. DNA was extracted from papain digested samples using phenol-chloroform and DNA content was determined by spectrophotometer readings at 260nm. C. GAG content varies with polymer composition. Biochemical samples were digested with papain and proteoglycan content was determined using the DMMB-dye method. Samples formed using high molecular weight microspheres were higher in GAG content than other groups, but were only statistically higher in GAG content than buffered microspheres after 8 weeks ($p < 0.02$). $n=2$ for buffered microsphere samples, $n=3$ for uncapped, 10 kDa microspheres samples, and $n=4$ for 50 kDa microsphere or capped microsphere groups.

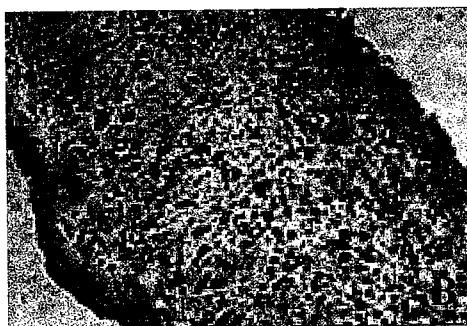


Figure 14. Immunostained sections for collagen types. Cartilage-like tissue made from cells alone (A, C) or cells and microspheres (B, D) was embedded in paraffin and sectioned. Sections were immunostained as described for collagen type I (A, B) or collagen type II (C, D). All samples exhibited positive staining for collagen type II, indicated by brown, and negative staining for collagen type I, as indicated by blue. Neither positive staining for collagen type I or negative staining for collagen type II was not observed in any samples.

	GAG (%ww ^e)	GAG/DNA (ug/ug)	DNA/tissue (ug/mg)
Chondocytes only	1.77%	41.5	0.96
PLG microspheres - high molecular weight	2.23%	114	0.61
Cartilage	5.18%	120	0.54
^e % ww - as percentage of wet weight			

Table 4. Summary of compositions and comparison with native bovine articular cartilage. Total GAG contents were normalized to either wet weight or DNA content and compared between cartilage formed from cells alone, cells and 50 kDa PLG microspheres, and native bovine cartilage tissue. Total DNA was normalized to tissue wet weight and compared between the groups.

CHAPTER IV

ENHANCEMENT OF CHONDROGENESIS THROUGH CONTROLLED RELEASE OF IGF-I FROM AN INJECTABLE MICROSPHERE SCAFFOLD

Summary

PLG microspheres have been used independently as a drug delivery vehicle or as a scaffold for cartilage tissue engineering. This study reports the simultaneous use of PLG microspheres as scaffold for cartilage regeneration and IGF-I controlled release vehicle and demonstrates that delivery through controlled release can enhance cartilage tissue growth. Release profiles for IGF-I doses from 0.0007% and 0.3% were evaluated in vitro and the effects of these doses on chondrogenesis were evaluated in vivo. Profiles demonstrated biphasic IGF-I release, with initial bursts of less than 13% and controlled release detecting release of 40% of the total IGF-I load at 6 weeks and continued detection at 8 weeks in all doses tested. Release of IGF-I to growing cartilage tissue resulted in dose-dependent increases in tissue mass, GAG, and collagen type II. IGF-I release increased proteoglycan accumulation in a dose-dependent manner at each time point, with the highest GAG stimulation exhibited at 8 weeks. In contrast, although IGF-I increased collagen type II accumulation at each time point, the stimulation of collagen

production was highest at 2 weeks and decreased with the duration of the study. Overall, the samples receiving 0.3% IGF-I exhibited the most tissue, GAG and collagen type II accumulation. Additionally, in samples receiving IGF-I, chondrocyte migration into and matrix deposition onto microspheres was dose-dependently enhanced at 4 weeks, with the most matrix deposition on microspheres occurring in samples receiving 0.3% IGF-I. The 0.3% IGF-I group also exhibited the most intense staining for proteoglycans and collagen type II after 8 weeks.

Introduction

The poor regenerative capacity of cartilage tissue and the current limitations of cartilage repair technologies make tissue engineering a promising repair alternative.⁸⁶ Tissue engineering strategies typically involve a polymer, cell source and/or growth factor, and studies of each of these factors have been of great interest for cartilage repair.

As a major component for tissue repair, the scaffold has been an integral area of study. As such, repair scaffolds have varied from pre-shaped biomaterials such as PLA, PGA or PLG^{20,21} to injectable and moldable materials such as alginate^{26,87} or fibrin.^{24,68,88} Varying success has been demonstrated with many types of polymers; however, PLG microspheres are an attractive scaffold for cartilage tissue engineering as they have been demonstrated to support cartilage growth as a solitary injectable scaffold in vivo or a moldable scaffold in vitro.^{82,166} PLG microspheres when incubated with chondrocytes have been shown to develop tissue volume in a constrained or molded system¹⁶⁶ or form cartilage nodules when injected subcutaneously,⁸² demonstrating that PLG microspheres support chondrogenesis.

Delivery of growth factors has also been shown to enhance cartilage tissue engineering and delivery has been demonstrated through a variety of methods. Growth factors are commonly added exogenously in vitro, yet these methods are cumbersome, as protein must be added frequently.²⁴ Additionally, strategies for exogenous delivery of growth factor in vivo are not straightforward, and as a result, development of alternative growth factor release methods is actively being explored. Transfection of chondrocytes with expression vectors encoding growth factor genes has exhibited promising results as an alternative method for growth factor delivery. Using this system, transfected chondrocytes have been seeded in polymer scaffolds⁷⁰ or transplanted onto cartilage disks.⁶⁹ Additionally, delivery of growth factor by controlled release from a polymer scaffold has also been investigated. Gel polymers such as fibrin or alginate can release free protein^{71,72} or encapsulate a delivery vehicle such as growth factor bound heparin-sepharose beads;⁷³ both methods demonstrated sustained release over an extended period of time.

Biodegradable polymers such as PLG have also been used for controlled release. One particularly suitable strategy for controlled delivery from PLG is in the form of microspheres, which have predominately been used as drug delivery vehicles.^{41,43,45} Recently, PLG microspheres have been used as a growth factor delivery vehicle in tissue engineering. PLG microspheres with encapsulated insulin-like growth factor-I (IGF-I) or transforming growth factor- β (TGF- β) were incorporated with chondrocytes in photopolymerizing gels. The effects of growth factor release in this system demonstrated increases in tissue and matrix production out to 2 weeks.²⁵ Thus, the promise of

controlled delivery combined with the tissue engineering applications of PLG microspheres suggest that microspheres could be used as a simultaneous repair scaffold and growth factor delivery vehicle.

One such growth factor that would be appropriately delivered from PLG microspheres for engineered cartilage tissue is IGF-I. IGF-I is an important growth factor for chondrogenesis, as it has been shown to stimulate matrix synthesis^{45,56,58,59} and cell proliferation^{58,60} and protects against matrix catabolism.⁵⁹ In tissue engineering applications, supplementation with IGF-I has demonstrated pronounced effects in vitro^{24,25,67} and in vivo.^{24,68} In addition, drug delivery studies have demonstrated stably loaded IGF-I into PLG microspheres for sustained delivery,⁷⁴ while controlled release in vivo has demonstrated increases of IGF-I serum levels in rats and mice.⁴⁵

Given that PLG microspheres can act as a solitary scaffold and have been demonstrated for use in controlled release, we sought to combine these functions to produce a simultaneous scaffold and growth factor delivery vehicle. We further hypothesized that release of IGF-I from PLG microspheres would enhance cartilage matrix assembly in engineered cartilage.

Materials and Methods

Cell Culture

Chondrocytes were isolated from the articular cartilage of calf gleno-humeral joints of 2-10 day old calves (Research 87, Hopkinton, MA) using 0.3% type II collagenase (Worthington), as previously described⁷⁶. After 16 hr of collagenase treatment at 37°C, isolated chondrocytes were filtered through a 180 µm filter to remove any undigested cartilage particles, washed several times with PBS, and cells were resuspended in Ham's F12 media supplemented with 1% ascorbic acid.

IGF-I protein preparation

To prepare the protein for microsphere encapsulation, recombinant human IGF-I protein (PeproTech, Inc, Rocky Hill, NJ) was dissolved in 100 mM solution of acetic acid for a 1 mg/mL solution. The protein solution was then sprayed through a sonic nozzle over liquid nitrogen, transferred to -80°C for nitrogen evaporation, and lyophilized.

Microsphere Preparation

PLG was obtained from Alkermes, Inc. PLG microspheres were fabricated using the ProLease[®] process, as described⁸⁹. Briefly, 50:50 lactide:glycolide polymer with a 50 kDa molecular weight and lyophilized IGF-I protein was dissolved in methylene chloride. This solution was atomized over a bed of liquid nitrogen layered on top of frozen ethanol. The temperature of the system was then maintained at -80°C to allow for nitrogen evaporation and removal of methylene chloride into the ethanol phase (microsphere curing). The resulting microspheres (loaded microspheres) were filtered and then lyophilized to remove residual ethanol, and finally sieved to obtain the desired range of

particle sizes. Microspheres of the same polymer type were prepared without IGF-I (unloaded microspheres), as described.⁸²

IGF-I load

As prepared, microspheres had a 1% IGF-I load. To obtain the desired dosage of IGF-I from 0.0007% to 0.3%, loaded microspheres and unloaded microspheres were mixed at different concentrations (Table 5).

IGF-I Release

Microspheres at varying IGF-I concentrations were incubated in vitro for up to 56 days in Ham's F12 media with 0.3% carboxymethylcellulose (Sigma Chemical Company, St. Louis, MO). At each time point, media was collected and replaced with fresh media. (n=3 for each effective IGF-I load).

Media was assayed for IGF-I content using the Human IGF-I Quantikine ELISA kit according to the manufacturers specifications (R&D Systems, Inc, Minneapolis, MN). IGF-I standards and collected media were incubated on an ELISA plate pre-coated with mouse monoclonal IGF-I antibody for 2 hr at 4°C. Unbound samples were washed from plate and the plate was incubated with an IGF-I polyclonal antibody conjugated to horseradish peroxidase at 4°C. Excess secondary antibody was washed from the plate and wells were incubated with hydrogen peroxide/chromogen for detection of IGF-I. Optical densities were read at 450nm.

Culture Conditions and Implantation

Chondrocytes were mixed with equal volume of PLG microspheres, and the microsphere-chondrocyte suspension was incubated for 4 hr at 37°C in a shaker incubator, as described.⁸²

Chondrocyte-microsphere suspensions were injected into the subcutaneous space of the dorsum of nude mice using an 18-gauge needle. Each injection contained 20×10^6 cells and/or 16 mg of microspheres in 500 μ L of F12 media (n=6 per group tested).

All animal procedures were performed under the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. Mice were sacrificed at 2, 4, and 8 weeks by anesthesia overdose and samples were harvested.

Sample Analysis

Upon harvest, the fibrous capsule was removed from each sample. The sample was first weighed and then cut in thirds. One-third of the sample was digested with 0.14 mg papain (Sigma Chemical Company) for 16 hr at 60°C. Papain digested samples were subjected to biochemical analysis for cell number and proteoglycan content. A second third of the sample was fixed in 10% formalin buffer. The fixed sample was embedded in paraffin and sectioned. Sections were stained with safranin-O. Unstained sections were probed for collagen type I and type II by immunohistochemistry, as described below. The last third of the sample was digested to extract the collagen molecules from the tissue and collagen II content was determined by ELISA as described below.

DNA Quantification

DNA quantification was performed as described.⁸² Briefly, papain digested samples mixed with phenol-chloroform-isoamyl alcohol (Sigma Chemical Co., St. Louis, MO). DNA was extracted and content was read by spectrophotometry at 260 nm and the purity was determined by the 260/280 ratio. A known number of chondrocytes was used as a standard for calculation of cell number.

Proteoglycan Content Analysis

Papain digested samples were analyzed for glycosaminoglycan (GAG) content using the DMMB-dye binding assay.⁷⁷ Briefly, 50 μ L of papain digested sample was incubated with 2 mL of DMMB-dye and the reaction was observed on a spectrophotometer at 525 nm, with shark chondroitin sulfate (Sigma Chemical Company) used as a standard.

Immunohistochemistry

Detection of collagen types I and II was achieved by immunohistochemical staining as described.⁸² Briefly, unstained paraffin sections were deparaffinized, rehydrated and washed in PBS with 0.1% BSA. Samples were incubated with 0.3% hydrogen peroxide and subjected to digestion for 30 min with 33U/mL bovine testicular hyaluronidase (Sigma Chemical Company). Sections were incubated with anti-collagen type I (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) or anti-collagen type II (Chemicon, Temecula,

CA) and incubated with anti-IgG biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). Sections were then subjected to avidin-biotin-peroxidase reagent (Vector Laboratories) and exposed to diaminobenzidine (Vector Laboratories). Sections were counterstained with 1% alcian blue for 3 min for collagen type I staining or 0.02% fast green for 4 min for collagen type II staining. Digital photomicrographs were obtained using a Nikon TE 200 microscope equipped with a Spot Junior camera (both from Micro Video Instruments, Avon, MA).

Collagen Extraction and ELISA

Collagen molecules were extracted from the tissue samples using methods described in the Native Type II Collagen Detection Kit #6009 (Chondrex, Inc., Redmond, WA). Briefly, tissue samples were incubated with 3M guanidine in 50 mM Tris-HCl buffer overnight at 4°C. The tissue was homogenized and the tissue precipitate was collected and washed with cold water. The precipitate was re-suspended in 800 µL of 0.5M NaCl in 50 mM acetic acid and digested with 1 mg pepsin (Worthington Biochemical Company) for 48 hr at 4°C. After pepsin digestion, 100 µL of 10x TSB and 0.1 mg of pancreatic elastase (Sigma Chemical Company) were added and samples were mixed overnight at 4°C. Samples were centrifuged and the supernatant was collected.

Collagen Type II ELISA

Collagen content was determined using the Native Type II Collagen Detection Kit #6009 according to the manufacturer's specifications (Chondrex, Inc). Briefly, the capture

antibody was allowed to adhere to the provided plate overnight. The antibody was removed and the plate was washed with wash buffer. Type II collagen was used as a standard and 100 μ l of standards and samples was incubated on the plate at room temperature for 2 hr. Standards and samples were removed, the plate was washed, and 100 μ l of detection antibody was incubated on the plate at room temperature for 2 hr. The detection antibody was removed, the plate was washed, and 50 μ l of streptavidin-peroxidase solution was added to the wells and incubated at room temperature for 1 hr. Upon removal of the streptavidin-peroxidase, the plate was washed and a solution of OPD chromogen and H_2O_2 Urea was allowed to react in each well for 30 min at room temperature. The reaction was stopped with 50 μ l of 2.5N sulfuric acid and the plate was read at 490 nm.

Statistical Analysis

All statistics were performed using Sigma StatTM Statistical Software Version 2.0. To detect statistical differences between tissue mass, proteoglycan accumulation or DNA content, two-way ANOVA tests were performed with post-hoc Bonferroni test for pairwise comparison.

Results

PLG microspheres exhibited typical release patterns with an initial IGF-I burst release followed by prolonged release. Most IGF-I microsphere dose samples (0.007%-

0.3%) had consistent release profiles beginning with low initial bursts of less than 9% and controlled release out to 8 weeks (Figure 16A). In the controlled delivery phase, the IGF-I in the media of these samples was detected as early as 1 week, with continued detection out to 8 weeks. In contrast, microspheres with an IGF-I concentration of 0.0007% exhibited a slightly larger percent release of total effective IGF-I protein during the initial burst phase and released a smaller percentage of IGF-I at 6 and 8 weeks than other doses (Figure 16A). When initial burst patterns were analyzed with respect to IGF-I release, an inverse relationship was observed. Similarly, the amount of IGF-I released to the media in the initial burst phase was highest from 0.0007% IGF microspheres and decreased as the percent IGF-I in the initial system increased (Figure 16B).

Cartilage samples increased in tissue mass over the 8 weeks in vivo. No significant differences between different dose groups were detected at earlier time points of 2 and 4 weeks. After 8 weeks, sample mass varied significantly with IGF-I dose. Cartilage formed with microspheres containing 0.3% IGF-I exhibited the highest tissue mass (Figure 17A), and these values were significantly higher than the lowest IGF-I conditions ($p < 0.01$).

Cell content was determined in all samples, but cell number did not vary between the different IGF-I conditions at any of the time points. Further, cell number was fairly comparable to the initial cell load of 20×10^6 cells at all of the time points in all of the conditions (Figure 17B).

GAG trends toward a dose response to IGF-I were detected at each time point, although no significant differences could be detected at weeks 2 or 4 (Figure 18A). After

8 weeks in culture, cartilage formed using 0.3% IGF-I microspheres exhibited significantly higher GAG content than cartilage from the 0.0007 or 0.007% IGF-I microsphere ($p < 0.02$). Normalization of GAG density at each time point to samples that did not receive IGF-I, revealed that the most pronounced dose dependent trends were present after 8 weeks (Figure 18B).

Proteoglycan distribution was assessed using safranin-O staining. Uniform staining across the tissue section was evident at all time points in all conditions (Figure 19). After week 2, proteoglycans were not deposited on microspheres and microsphere integrity was not compromised (Figure 19A-C). The beginnings of microsphere degradation were evident after 4 weeks in all samples (19D-F), but staining on microspheres differed. In samples formed from 0% through 0.07% IGF-I, microspheres exhibited slight staining for proteoglycan deposits (Figure 19D,E and data not shown). In contrast, evidence of GAG deposition on microspheres appeared to be increased in 0.3% samples. Moreover, these samples indicated increased chondrocyte migration into the microspheres (Figure 19F). After 8 weeks, proteoglycan staining was uniform across all samples after 8 weeks and detection of microsphere remained was difficult to detect (Figure 19G-I). Staining across these samples appeared most intense in cartilage formed with microspheres containing 0.3% IGF-I at all time points (Figure 19C,F,I), whereas cartilage formed using other doses of IGF-I or no IGF did not vary in intensity (Figure 19A,B,D,E,G,H).

Dose responses to IGF-I were detected at weeks 2 or 4, but after 8 weeks in culture a well defined trend in dose response was evident. After 8 weeks, samples

receiving the highest amounts of IGF-I exhibited increased collagen type II content, and cartilage formed with 0.3% IGF-I microspheres had significantly higher collagen content than the lowest IGF-I doses ($p < 0.02$) (Figure 20A). When collagen type II per wet weight was normalized to data from samples without IGF-I, analyses indicated that stimulatory increases in collagen type II were highest at week 2 but gradually decreased over the 8 weeks (Figure 20B).

Collagen content was also detected via immunohistochemistry for collagen type I or collagen type II. All samples stained negative for collagen type I at each time point (Figure 21A,B) and positive for collagen type II at all time points (Figure 21C-F and data not shown). After 8 weeks, a gradient of staining intensity in cartilage sections with differing IGF-I microspheres was detected. Specifically, as the percentage of IGF-I in the system increased, cartilage sections exhibited stronger positive staining for collagen type II (Figure 21C-F).

Discussion

Prior work has independently demonstrated PLG microspheres as a scaffold for tissue engineering and as a vehicle for controlled release of growth factors. This study demonstrates, for the first time to our knowledge, that PLG microspheres can be used as a simultaneous tissue engineering scaffold and vehicle for controlled growth factor delivery. Further, this study demonstrates long-term enhancement of chondrogenesis by controlled release of IGF-I doses *in vivo*.

Protein release via controlled delivery is an important concept for tissue engineering, as exogenous delivery may be limited by short protein half lives⁹⁰ or interactions with binding proteins and diffusion through ECM.⁹¹ Further, administering growth factor directly in vivo would require multiple deliveries, possibly through intra-articular injection⁹² or topical application.⁹³ Controlled delivery of growth factor offers a viable alternative and an easy delivery model for the inclusion of growth factors for repair by tissue engineering.

Determining release kinetics is a necessary first step in evaluating the effectiveness of controlled delivery. Typical release patterns from microspheres or microparticles have indicated a bi-phasic release profile.^{44,47,48,94} In the first phase, referred to as initial burst, immediate release occurs by diffusion of growth factor tethered to the surface of the microsphere. The second phase of release results in sustained delivery of growth factor attributed to polymeric degradation, and the length of this phase can be controlled by the properties of the polymer.^{23,82,95}

Much work has been done to lower the initial burst exhibited in controlled release studies, as significant early loss of protein will decrease the amount of protein available for sustained release. Work to control initial burst has suggested that the addition of excipient factors may lower initial burst in some systems.⁷⁴ However, this study did not require the addition of excipient to control initial burst, and results indicated that initial bursts of IGF-I from PLG microsphere fell between 4-13%. The release profiles from microspheres of the various IGF-I doses suggested a trend that initial burst decreased with increasing IGF-I dose. This relationship between initial burst and IGF-I dose,

although interesting, was not determined for statistical significance and would bear further investigation in order to draw any more conclusions.

Previous studies have demonstrated prolonged IGF-I release out to 4 weeks in an in vitro drug delivery study⁴⁷ and out to 2 weeks in a tissue engineering application.²⁵ In this study, IGF was detected out to 8 weeks. Sustained release profiles for this study indicated phase II release initiating at 1 week and steadily increasing to peak release at 6 weeks, with continued detection after 8 weeks. While total protein released after 8 weeks fell between 65-75% of the initial IGF-I in the system for most doses, virtually complete protein release from microspheres has been demonstrated in microspheres fabricated using the ProLease[®] process,⁴⁶ suggesting that controlled release of IGF-I in our system may continue beyond 8 weeks.

In analyzing these samples by histology, the observed degradation trends of the microspheres in IGF-I samples were fairly consistent with previous results⁸². Typically for ProLease[®] microspheres made with 50 kDa polymer, evidence of microsphere degradation was not observed before 4 weeks (Chapter II, III). Similarly, matrix deposition in this study remained excluded from microspheres at 2 weeks, and after 8 weeks matrix covered the majority of microsphere area. However, noticeable differences were detected at 4 weeks, as variations in matrix deposition were exhibited. Specifically, degradation had occurred in many of the microspheres in all samples, but matrix deposition on microspheres was markedly higher in 0.3% IGF-I samples. Two possible explanations exist for this observation. First, as IGF-I diffusion can be stunted by interactions with matrix molecules such as IGF binding proteins,⁹¹ cells closest to the

microsphere may come into contact with more IGF-I than chondrocytes more distant from microspheres. Second, IGF-I has been implicated in chemotactic increases in meniscal fibrochondrocytes and chondrocyte motility.^{28,96} Subsequently, higher IGF-I doses may increase the stimulation of chondrocyte migration into microsphere areas, allowing them to make and deposit more matrix in these locations.

Similar to microsphere degradation patterns, the extent of tissue and matrix accumulation was consistent with release profiles. Cartilage samples increased in tissue size and matrix deposition at 2 and 4 weeks, but the most pronounced effects were exhibited at 8 weeks for each dose, which is likely an effect from IGF-I released at 6 weeks. At early time points (2 and 4 weeks) a trend toward IGF-I dose-dependent response was displayed in total matrix accumulation, yet no significant differences were detected. After 8 weeks, however, a pronounced dose-dependent effect from IGF-I was exhibited, with the highest dose of IGF-I correlating with the highest mass, GAG accumulation and collagen content. Similarly, these effects were demonstrated by staining intensity for proteoglycans (Figure 19) and collagen type II (Figure 21), as sections from 0.3% IGF cartilage exhibited the deepest stain.

Although overall content detection demonstrated increased GAG and collagen type II accumulation over 8 weeks (Figure 18 and Figure 20) and subsequent normalization of these data to 0% IGF-I samples also exhibited increases in overall content, analysis of normalized densities of proteoglycan or collagen type II (Table 6) suggests differential regulation of these matrix molecules. Analysis of matrix accumulation to determine the maximum amount of stimulation and the dose necessary to

elicit half maximal response (ED_{50}) indicated that stimulation of collagen type II was maximal after 2 weeks, but this stimulation consequently decreased at 4 weeks and again at 8 weeks (Table 6). ED_{50} was also higher at 8 weeks, indicating that more IGF-I was required to elicit an effective response. In contrast, stimulation of GAG density was minimal at 2 weeks, but increased with time (Table 6), while ED_{50} decreased by a factor of 100 from 2 to 8 weeks. The overall findings of dose-dependent increases in GAG and collagen type II content in this study are consistent with matrix molecule analysis of other studies where intact cartilage or tissue engineered constructs were subjected to IGF-I.^{24,60,61,68} However, no suggestion of differential regulation of collagen and proteoglycan accumulation has been reported in these studies. IGF-I control of matrix molecules is believed to be at the level of transcription.¹⁶³ However, in this study we looked at the effective accumulation of translated product; thus an understanding of the transcript levels of these matrix molecules in a controlled release system may give insight into this phenomenon of differential regulation.

Another factor to be considered in a controlled release scaffold system is the effective dose of growth factor, and typical effective doses of IGF-I for tissue engineering have ranged between 10 ng/mL and 300 ng/mL.^{24,60,61,67} As the controlled release system does not deliver consistent amounts of protein over a given time period, the effective doses may be different from exogenous delivery concentrations used in other studies. Therefore, we evaluated a range of IGF-I doses spanning roughly 3.5 orders of magnitude. The different IGF-I doses all increased tissue mass and GAG and collagen accumulation over the duration of the study. However, dose-dependent increases in

tissue components suggested that the dose eliciting the highest tissue and ECM accumulation was 0.3% IGF, corresponding to 48 μ g of total initial protein. Although physiological levels of IGF-I have been shown to be effective in vitro, supraphysiologic levels of IGF-I do not have negative effects and can still stimulate matrix growth.⁶⁸ Further, in a controlled release system, high levels of growth factor delivery may be needed for a maximal stimulatory effect as IGFBP binding or diffusion of IGF away from the cartilage implant may impact the effectiveness of the protein by making it unavailable to the IGF receptor.

Although the effective dose may be different for a controlled release system, the doses used in this study demonstrate consistency with previous studies that have shown dose-dependent responses to IGF-I stimulation of extracellular matrix molecule synthesis.²⁴ However, there are varying reports on the effects of IGF-I on cell proliferation. Some studies have demonstrated increases in DNA content,⁶⁰ while others have not seen enhanced DNA synthesis with IGF-I supplementation.²⁴ Our data was found to be consistent with studies that do not demonstrate dose-dependent effects on cell proliferation.

Overall, this study demonstrates long-term, effective controlled release of IGF-I protein from PLG microspheres. The controlled release of IGF-I increased tissue and ECM accumulation in a dose-dependent manner. Thus, controlled release of proteins offers an effective and easy means of incorporating growth factors into a tissue engineering design with applications for enhanced matrix and tissue output through prolonged and controlled exposure of cells to growth factors.

Acknowledgements

The authors would like to thank Kristin Prinn for her help with microsphere fabrication.

IGF-I Dose	Total IGF in system (ug)	ng IGF-I released/mg tissue (8 wk)
0%	0	0
0.0007%	0.112	2.17
0.007%	1.12	4.48
0.02%	3.2	10.31
0.07%	11.2	28.57
0.3%	48	86.3

Table 5. Total IGF-I protein loaded for each dose condition. PLG microsphere with a 1% IGF-I load were fabricated using the ProLease® process. Loaded and unloaded microspheres were mixed in different ratios to obtain a range of IGF-I effective loads between 0 and 0.3% IGF-I. Total protein content in 16 mg of PLG microspheres are given, and total amounts of IGF-I released at each dose was normalized to sample mass at 8 weeks to give an understanding of IGF-I doses.

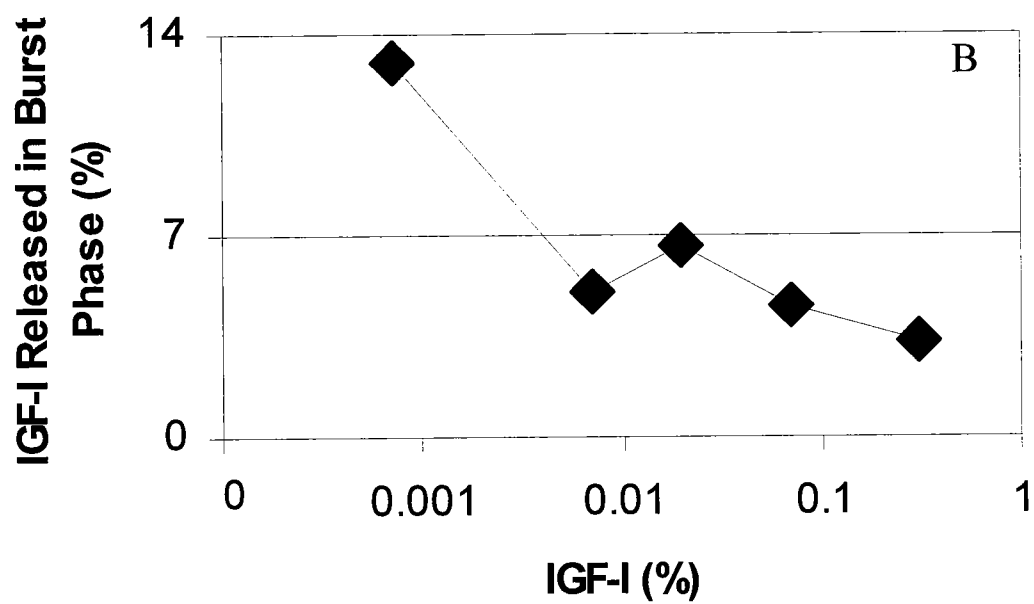
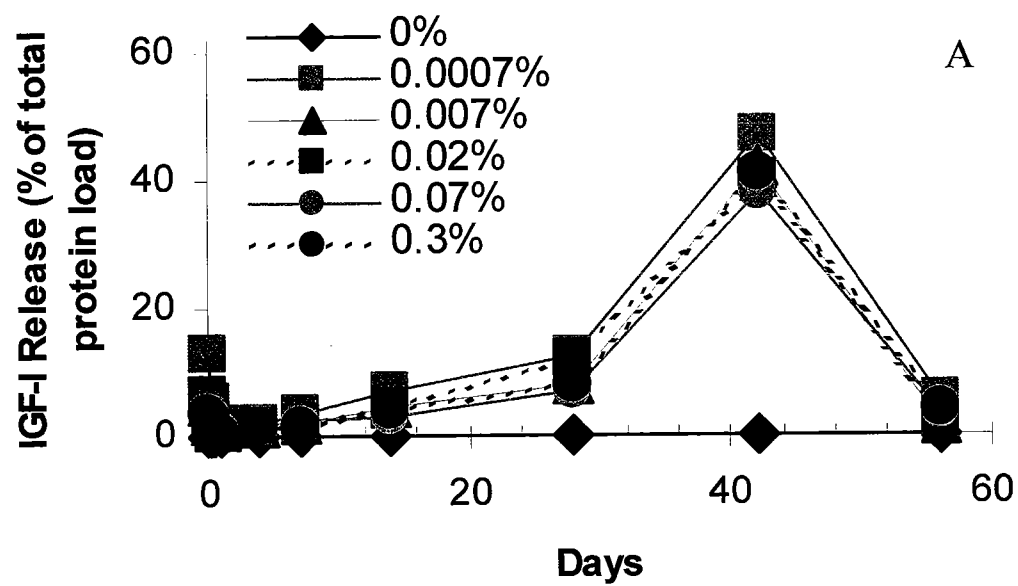


Figure 16. Release profiles were consistent between different doses of IGF-I released from PLG microspheres with loads ranging from 0.0007% to 0.3% (g of IGF-I per g of microspheres). Microspheres were incubated in vitro in media and the supernatant was collected at time points between 0 and 56 days. IGF-I concentration in the media was detected by ELISA and normalized to total IGF-I loaded in the microspheres. A. IGF-I release profile of each dose over 8 weeks indicated bi-phasic release patterns. Supernatant concentrations from 0 days correlated to initial burst, while samples taken from 4 hours to 56 days indicated the sustained release phase. B. Amount of IGF-I released in initial burst decreases with increasing IGF-I dose. IGF-I concentrations from 0 days (initial burst samples) were plotted against IGF-I dose.

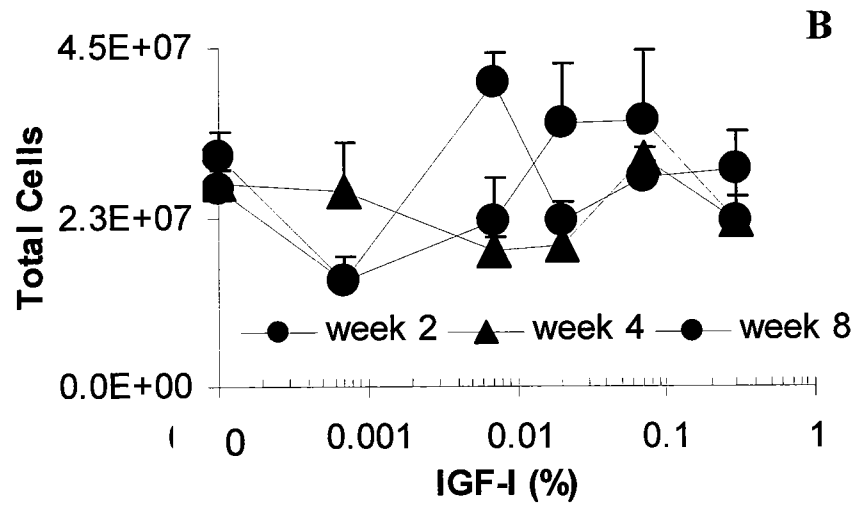
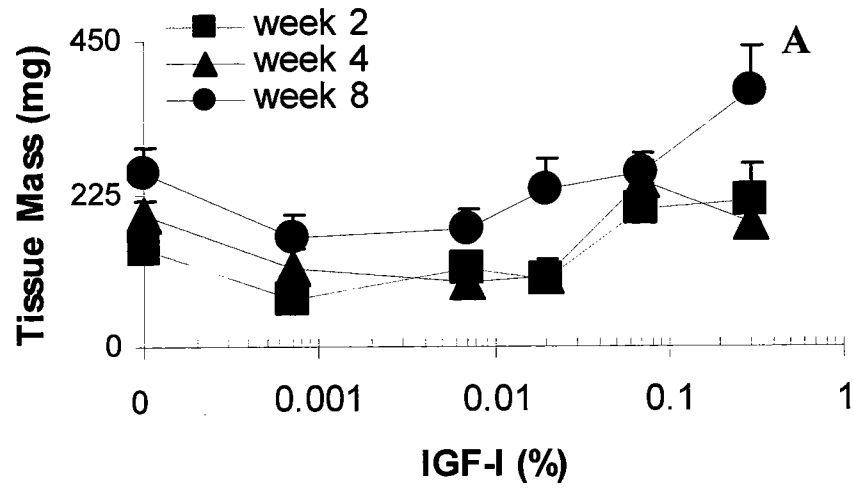


Figure 17. Tissue engineered cartilage responded to controlled release of IGF-I.

Controlled release of IGF-I induced a dose-dependent increase in tissue mass at all time points (A), but not cell content (B). $n=6$ for all groups.

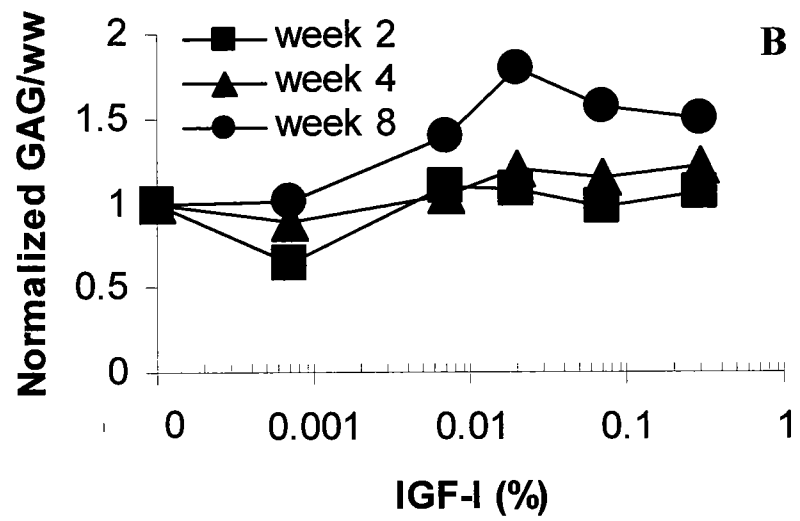
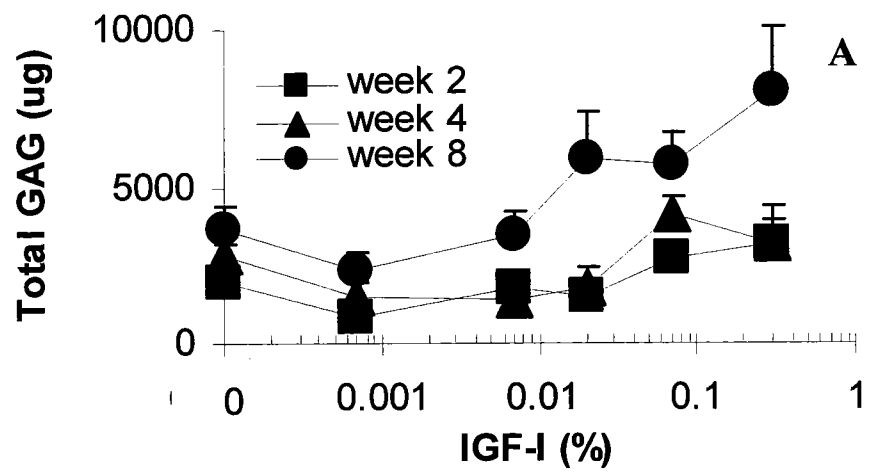


Figure 18. Biochemical analysis of IGF-I effects on proteoglycan content in tissue engineered cartilage. DMMB dye was used as a colorimetric readout for proteoglycan content. A. Controlled IGF-I release increased proteoglycan content over time in a dose-dependent manner at each time point, with samples receiving 0.3% IGF-I exhibiting the highest GAG content after 8 weeks. B. GAG density (GAG/ tissue wet weight) normalized to samples without IGF-I was highest at 8 weeks in all samples.

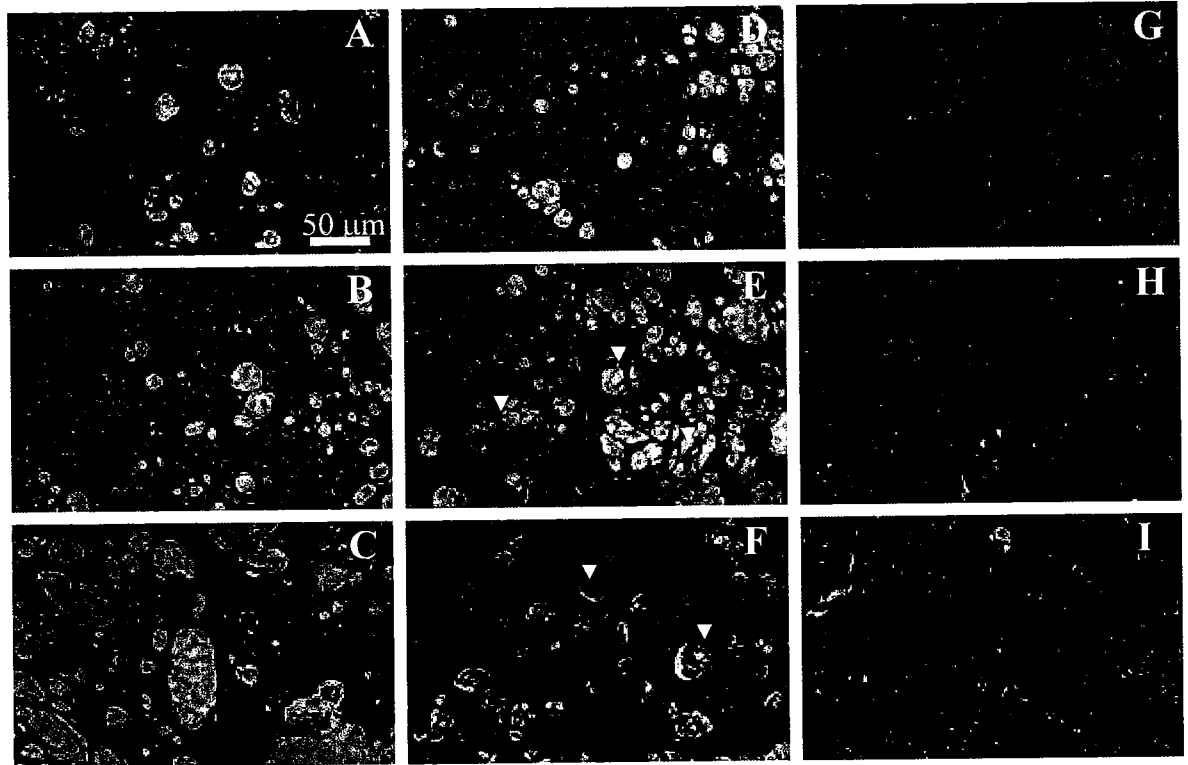


Figure 19. Proteoglycan staining intensity increased with IGF-I dose. Cartilage sections stained with safranin-O from 2 week samples, 0% (A), 0.07% (B), or 0.3% (C), or from 4 week samples, 0% (D), 0.007% (E), 0.3% (F), indicated the deepest staining in 0.3% IGF-I sections at each time point. No differences in staining intensity were detected at these time points for lower doses of IGF-I and samples without IGF-I. At 4 weeks, samples receiving controlled release of IGF-I exhibited increased matrix deposition onto the microspheres, with microspheres from 0.3% samples exhibiting the most matrix deposition. (Matrix deposition on the microspheres at 4 weeks is indicated by yellow arrowheads). Cartilage sections from 8 week samples, 0% (G), 0.02% (H), and 0.3% (I), exhibited increased staining intensity with increasing IGF-I dose. All pictures are magnified 200x.

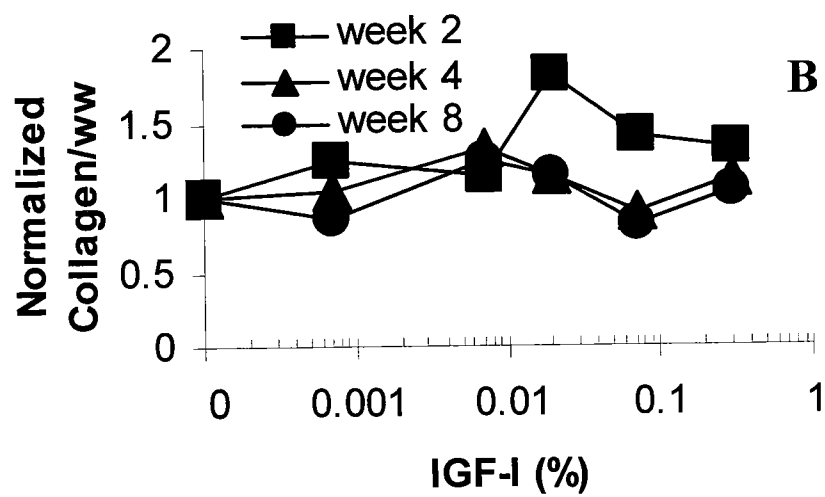
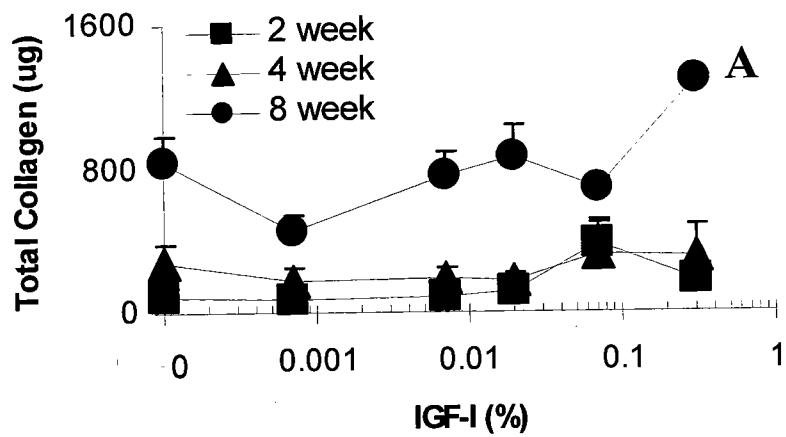


Figure 20. IGF-I induced dose-dependent increases in collagen type II content. ELISA was used to determine total collagen type II in all samples, as described in materials and methods. A. IGF-I induced dose-dependent increases in total collagen type II accumulation at all time points. The most pronounced dose-dependent effects were observed at 8 weeks, with the highest collagen type II content found in 0.3% samples. B. Collagen type II density (collagen/wet weight) normalized to samples without IGF-I at each time point indicated dose-dependent increases in collagen type II density only at 2 weeks. After 4 or 8 weeks, collagen density did not differ by dose or by the addition of IGF-I in the system.

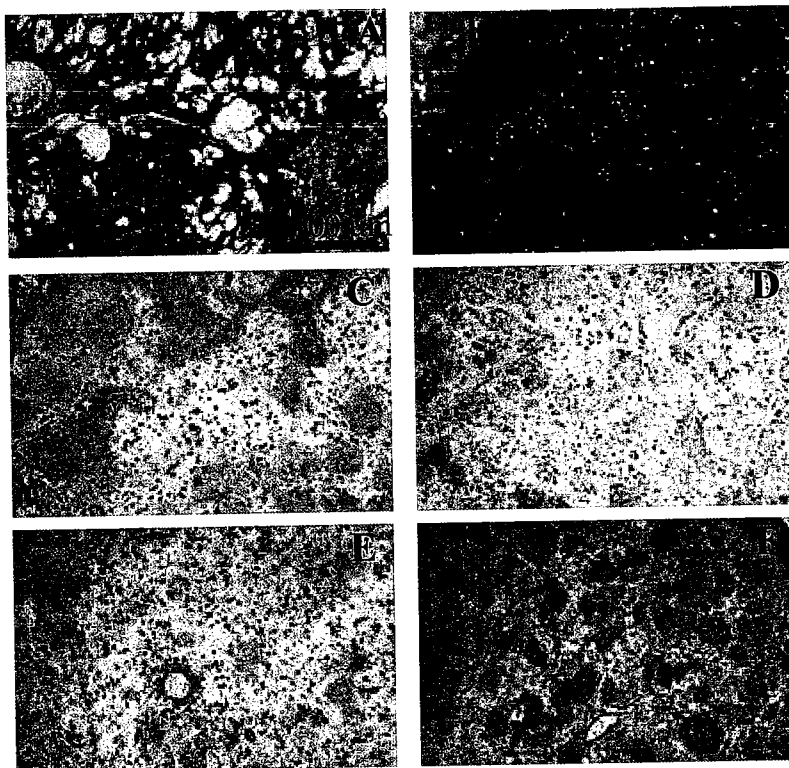


Figure 21. Immunohistochemical staining for collagen types I and II. All samples indicated were negative for collagen type I, as indicated by blue, at 2 weeks (A) or 8 weeks (B) and positive staining for collagen type II, as indicated by brown (C-F). Samples stained for collagen type II at 8 weeks, 0% (C), 0.0007% (D), 0.02% (E), and 0.3% (F), indicated dose-dependent increases in staining intensity. All pictures are magnified 100x.

Time (wk)	0.3% IGF-I GAG/ww (ug/mg)	0.3% IGF-I Col II/ww (ug/mg)	Max GAG Stimulation	Max Col II Stimulation	GAG ED ₅₀ (%)	Col II ED ₅₀ (%)
2	14.7	0.82	1.08	1.47	0.27388	0.00098
4	17.6	1.67	1.22	1.16	0.01222	0.00085
8	21.5	3.46	1.63	1.04	0.00314	0.00154

Table 6. Differential regulation of matrix accumulation by controlled release of IGF-I. GAG and collagen type II content at 0.3% IGF-I increased at each time point, however, the relative stimulation of GAG and collagen type II, as normalized to 0% IGF-I indicated differential regulation. The effective dose necessary to elicit 50% maximal response (ED_{50}) at each time point was calculated.

CHAPTER V

UTILITY OF PLG MICROSPHERES CONTAINING IGF-I FOR IN VITRO CARTILAGE TISSUE ENGINEERING

Summary

PLG microspheres can be used as a scaffold for cartilage tissue engineering and have been used in vivo as a simultaneous growth factor delivery vehicle. This study demonstrates the utility of controlled release of IGF-I from PLG microspheres on in vitro chondrogenesis and that this method can be used as an alternative delivery method to exogenous IGF-I addition in cartilage tissue engineering designs. We demonstrated that IGF-I induced a dose-dependent increase in both GAG and collagen type II, but no dose-dependent changes were exhibited in tissue mass or cell number. Moreover, the effective dose for enhanced tissue formation was 0.02% IGF-I. At IGF-I effective loads higher than 0.02%, a decrease in GAG and collagen type II accumulation was observed. All tissue sections exhibited IGFBP-2 and IGFBP-6, which could play a role in the decreased ECM accumulation detected at the highest IGF-I doses.

Introduction

The dual role of PLG microspheres as a polymer scaffold for cartilage tissue engineering and a simultaneous delivery vehicle for the growth factor IGF-I in vivo has

been presented. However, optimal scaffolds for cartilage tissue engineering will have utility both in vivo and in vitro. Understanding the nature of the in vivo environment is obvious, as these systems will replace tissue defects caused by trauma, age degeneration, or disease. However, in vitro culture conditions allow for the identification of specific factors that may affect chondrogenesis without confounding effects that can result from interactions with the host environment. In vitro evaluation of chondrogenesis is particularly important in a simultaneous controlled delivery and scaffold system, as it permits the study of many variables that can affect cartilage regeneration including the efficacy of growth factor release and the actions of the growth factor itself.

Drug delivery by PLG microspheres is known to be affected by many parameters. Microsphere size,⁹⁷⁻¹⁰⁰ variations in the fabrication process,^{101,102} or modifications in parameters that influence polymer degradation^{23,100,103} have all been demonstrated to affect drug delivery. Such parameters may exacerbate differences in drug delivery patterns under in vitro or in vivo conditions, and in fact one such parameter, microsphere degradation, has been shown to differ under these culture conditions; microspheres degrade roughly 2-fold slower in vitro.²³ Drug release in such systems follows a biphasic curve, with the second phase of drug release attributed to microsphere degradation.^{41,93} The impact of microsphere degradation in the in vitro environment could prolong the release of IGF-I to chondrocytes, which may translate into changes in cartilage matrix accumulation.

In addition to changes on drug release by the in vitro environment, the actions of the delivered growth factor must be understood. In cartilage biology, in vitro evaluation

becomes particularly important as it involves complex tissue homeostasis maintained by a balance of growth factors that can induce similar effects. Growth factors such as IGF-I and TGF- β stimulate the synthesis of proteoglycan and collagen molecules,^{56,58,60} and FGF, IGF-I and TGF- β also increase cell proliferation.^{58,60,103} Not only have multiple growth factors been shown to present similar stimulation of extracellular matrix (ECM) proteins, but feedback mechanisms can also be activated by multiple growth factors. For instance, both TGF- β and IGF-I can increase the concentration of IGFBPs in the tissue,^{104,105} and the increase in IGFBP production will subsequently elicit effects on IGF-I signaling. In some instances this feedback can inhibit IGF-I from binding to its receptor or facilitate IGF-I signaling in cells.⁵⁷ While it is difficult to understand the overlapping and duplicated actions of these growth factors in vivo, in vitro culture can be used to define the actions of a single factor and decouple its mediation of cartilage repair.

We have previously shown that PLG microspheres can be used for cartilage tissue engineering as a moldable scaffold in vitro. These cartilage tissue disks maintain thickness and shape, as well as increase the overall matrix and tissue accumulation. In this study, we expected that PLG microspheres could be used concurrently as both a scaffold and delivery vehicle to form shaped cartilage in vitro.

Materials and Methods

Cell Culture

Chondrocytes were isolated from the articular cartilage of calf gleno-humeral joints of 2-10 day old calves (Research 87, Hopkinton, MA) using 0.3% type II collagenase

(Worthington), as previously described.⁷⁶ After 16 hr of collagenase treatment at 37°C, isolated chondrocytes were filtered through a 180 µm filter to remove any undigested cartilage particles, washed several times with PBS, and cells were resuspended in Ham's F12 media supplemented with 1% ascorbic acid and 10% FBS.

IGF-I protein preparation

To prepare the protein for microsphere encapsulation, recombinant human IGF-I protein (PeproTech, Inc, Rocky Hill, NJ) was dissolved in 100 mM solution of acetic acid for a 1 mg/mL solution. The protein solution was then sprayed through a sonic nozzle over liquid nitrogen, transferred to -80°C for nitrogen evaporation, and lyophilized.

Microsphere Preparation

PLG was obtained from Alkermes, Inc. PLG microspheres were fabricated using the ProLease[®] process, as described.⁸⁹ Briefly, 50:50 lactide:glycolide polymer with a 50 kDa molecular weight and lyophilized IGF-I protein was dissolved in methylene chloride. This solution was atomized over a bed of liquid nitrogen layered on top of frozen ethanol. The temperature of the system was then maintained at -80°C to allow for nitrogen evaporation and removal of methylene chloride into the ethanol phase (microsphere curing). The resulting microspheres (loaded microspheres) were filtered and then lyophilized to remove residual ethanol, and finally sieved to obtain the desired range of particle sizes. Microspheres of the same polymer type were prepared without IGF-I (unloaded microspheres) using the process above.

IGF-I load

As prepared, microspheres had a 1% IGF-I load. To obtain the desired dosage of IGF-I, loaded microspheres and unloaded microspheres were mixed at different concentrations (Table 7).

IGF-I Release

Microspheres at varying IGF-I concentrations were incubated in vitro for up to 56 days in Ham's F12 media with 0.3% carboxymethylcellulose (Sigma Chemical Company, St. Louis, MO). At each time point, media was collected and replaced with fresh media.

Media was assayed for IGF-I content using the Human IGF-I Quantikine ELISA kit according to the manufacturers specifications (R&D Systems, Inc, Minneapolis, MN). IGF-I standards and collected media were incubated on an ELISA plate pre-coated with mouse monoclonal IGF-I antibody for 2 hr at 4°C. Unbound samples were washed from plate and the plate was incubated with an IGF-I polyclonal antibody conjugated to horseradish peroxidase at 4°C. Excess secondary antibody was washed from the plate and wells were incubated with hydrogen peroxide/chromogen for detection of IGF-I. Optical densities were read at 450nm.

Culture Conditions and Implantation

Chondrocytes, at a density of 80×10^6 cells/mL, were mixed with an equal volume of PLG microspheres, at a concentration of 64 mg/mL. The microsphere-chondrocyte suspension was incubated for 4 hrs at 37°C in a shaker incubator to allow for chondrocyte attachment to the microspheres.

For in vitro culture, the wells of a 12-well plate were covered with a surface layer of 0.5% agarose to prevent cell adhesion to the bottom of the well, and a cylinder measuring 1 cm in diameter was placed in the well. Agarose solidification sealed the cylinder to the well. Chondrocytes and microspheres were delivered as a 500 μ L volume to the cylinders. Each delivery contained 20×10^6 cells and 16 mg of microspheres in 500 μ L of F12 media (n=3-6 per group tested).

Sample Analysis

Upon harvest, each sample was first weighed and then cut in thirds. One-third of the sample was digested with 0.14 mg papain (Sigma Chemical Company) for 16 h at 60°C. Papain digested samples were subjected to biochemical analysis for cell number and proteoglycan content. A second third of the sample was fixed in 10% formalin buffer. The fixed sample was embedded in paraffin and sectioned. Sections were stained with safranin-O. Unstained sections were probed for collagen type I and type II by immunohistochemistry, as described below. The last third of the sample was digested to extract the collagen molecules from the tissue and collagen II content was determined by ELISA as described below.

DNA Quantification

DNA quantification was performed as described.⁸² Briefly, papain digested samples mixed with phenol-chloroform-isoamyl alcohol (Sigma Chemical Co., St. Louis, MO). DNA was extracted and content was read by spectrophotometry at 260 nm and the purity was determined by the 260/280 ratio. A known number of chondrocytes was used as a standard for calculation of cell number.

Proteoglycan Content Analysis

Papain digested samples were analyzed for glycosaminoglycan (GAG) content using the DMMB-dye binding assay⁷⁷. Briefly, 50 μ L of papain digested sample was incubated with 2 mL of DMMB-dye and the reaction was observed on a spectrophotometer at 525 nm, with shark chondroitin sulfate (Sigma Chemical Company) used as a standard.

Collagen Immunohistochemistry

Detection of collagen types I and II was achieved by immunohistochemical staining as described.⁸² Briefly, unstained paraffin sections were deparaffinized, rehydrated and washed in PBS with 0.1% BSA. Samples were incubated with 0.3% hydrogen peroxide and subjected to digestion for 30 min with 33U/mL bovine testicular hyaluronidase (Sigma Chemical Company) and blocked with 1.5% normal rabbit serum (Vector Laboratories, Burlingame, CA). Sections were incubated with a 1:50 dilution of anti-collagen type I (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) for 48 h at 4°C or a 1:10

dilution of anti-collagen type II (Chemicon, Temecula, CA) for 2 hr at room temperature, washed, and incubated with a 1:200 dilution of anti-IgG biotinylated secondary antibody (Vector Laboratories) for 30 min at room temperature. Sections were washed and subjected to avidin-biotin-peroxidase reagent (Vector Laboratories), washed, and exposed to diaminobenzidine (Vector Laboratories) for 2 min. Sections were counterstained with 1% alcian blue for 3 min for collagen type I staining or 0.02% Fast Green for 4 min for collagen type II staining. Digital photomicrographs were obtained using a Nikon TE 200 microscope equipped with a Spot Junior camera (both from Micro Video Instruments, Avon, MA).

Collagen Extraction

Collagen molecules were extracted from the tissue samples using methods described in the Native Type II Collagen Detection Kit #6009 (Chondrex, Inc., Redmond, WA). Briefly, tissue samples were incubated with 3M guanidine in 50 mM Tris-HCl buffer overnight at 4°C. The tissue was homogenized and the tissue precipitate was collected and washed with cold water. The precipitate was re-suspended in 800 µL of 0.5M NaCl in 50 mM acetic acid and digested with 1 mg pepsin (Worthington Biochemical Company) for 48 hr at 4°C. After pepsin digestion, 100 µL of 10x TSB and 0.1 mg of pancreatic elastase (Sigma Chemical Company) were added and samples were mixed overnight at 4°C. Samples were centrifuged and the supernatant was collected.

Collagen Type II ELISA

Collagen content was determined using the Native Type II Collagen Detection Kit #6009 according to the manufacturer's specifications (Chondrex, Inc). Briefly, the capture antibody was allowed to adhere to the provided plate overnight. The antibody was removed and the plate was washed with wash buffer. Type II collagen was used as a standard and 100 µl of standards and samples was incubated on the plate at room temperature for 2 hr. Standards and samples were removed, the plate was washed, and 100 µl of detection antibody was incubated on the plate at room temperature for 2 hr. The detection antibody was removed, the plate was washed, and 50 µl of streptavidin-peroxidase solution was added to the wells and incubated at room temperature for 1 hr. Upon removal of the streptavidin-peroxidase, the plate was washed and a solution of OPD chromogen and H₂O₂ Urea was allowed to react in each well for 30 min at room temperature. The reaction was stopped with 50 µl of 2.5N sulfuric acid and the plate was read at 490 nm.

IGFBP Immunohistochemistry

Unstained paraffin sections were deparaffinized for 5 min by two separate incubations with xylene. Samples were then rehydrated in 2 washes for 2 min each with 100% ethanol, 95% ethanol, and 80% ethanol, followed by 3 washes with PBS/0.1% BSA for 5 min each. Samples were incubated with 0.3% hydrogen peroxide and subjected to digestion for 60 min with 0.02U/mL chondroitinase ABC (Sigma Chemical Company) and blocked for 60 min with 1.5% normal goat serum (Vector Laboratories). Sections

were incubated with a 1:50 dilution of anti-IGFBP-2 (Upstate Biotechnology) or anti-IGFBP-6 (GroPep, Inc., Adelaide, South Australia) or an antibody:antigen complex for 2 hr at room temp. Antibody:antigen complexes were made 1 hr prior to incubation with sections and allowed to sit at room temperature during this time. To block IGFBP-2, the antibody:antigen complex was made using IGFBP-2 protein (GroPep, Inc.) in 10-fold excess of anti-IGFBP-2 by volume, and to block IGFBP-6, the antibody:antigen complex was made using 12 µg IGFBP-6 protein (GroPep, Inc.) to 2.5 µg anti-IGFBP-6. Sections were washed and incubated with a 1:200 dilution of anti-IgG biotinylated secondary antibody (Vector Laboratories) for 30 min at room temperature. Sections were washed and subjected to avidin-biotin-peroxidase reagent (Vector Laboratories), washed, and exposed to diaminobenzidine (Vector Laboratories) for 2 min. Sections were counterstained with Hematoxylin QS (Vector Laboratories) for 45 sec, and the counterstain was rinsed off with deionized water for 10 sec. Digital photomicrographs were obtained using a Nikon TE 200 microscope equipped with a Spot Junior camera (both from Micro Video Instruments).

Statistical Analysis

All statistics were performed using Sigma StatTM Statistical Software Version 2.0. To detect statistical differences between tissue mass, proteoglycan accumulation or DNA content, two-way ANOVA tests were performed with post-hoc Bonferroni test for pairwise comparison.

Results

IGF-I release was evaluated in microspheres with 0.0007% to 0.03% IGF-I. Total protein loads ranged from 0-48 μ g of IGF-I in the system (Figure 21A). Microspheres containing 0.007% to 0.3% IGF-I exhibited 65-75% growth factor release, while 0.0007% IGF-I microspheres released almost 100% of the growth factor (Figure 22B). In all cases, however, 50% of the IGF-I in the system was released by 29 to 38 days (Figure 22A).

Cartilage tissue was formed in the absence of IGF-I or in the presence of IGF-I either by exogenous delivery or controlled delivery. In most cases, the cartilage tissue maintained the disk-like shape provided by the mold, as demonstrated in our previous *in vitro* studies. However, at the two week time point, samples formed from PLG microspheres with 0.0007% IGF-I did not solidify into a disk and broke apart upon removal from the mold, and thus these samples could not be analyzed.

Cartilage growth supplemented with 30 ng/ml IGF-I by exogenous delivery or 0.02% IGF-I by controlled release overall formed similar cartilage tissue and both were significantly different from samples that did not receive IGF-I after 8 weeks for most parameters. Samples treated with exogenous IGF-I increased tissue mass over 8 weeks of culture, while samples formed with controlled delivery of IGF-I or no IGF-I maintained relatively constant levels of tissue mass (Figure 23A). No differences in cell number were detected between any groups (Figure 23B). In addition, more proteoglycans (Figure 23C) and total collagen II (Figure 23D) were accumulated in samples receiving IGF-I by both delivery methods when compared to samples that did not receive IGF-I. However,

no differences in these parameters were observed after 8 weeks between samples receiving IGF-I by exogenous addition or controlled delivery.

Samples receiving IGF-I through controlled delivery were then analyzed at IGF-I doses from 0%-0.7% of the total microsphere mass. In many of these samples tissue mass peaked by week 4 and by 8 weeks did not vary. No dose response to tissue mass was observed at any of the time points taken (Figure 24A). Similarly, cell number did not respond to IGF-I in a dose dependent manner, and overall, cell number remained unchanged throughout the study (Figure 24B).

However, dose responses to IGF-I were observed in samples analyzed for proteoglycan accumulation (Figure 25A). Without IGF-I or at the 0.0007% of IGF-I, proteoglycan accumulation was low and remained relatively unchanged through the 8 week study. Although all samples exhibited increased GAG content after 8 weeks ($p < 0.05$), dose responses to IGF-I were only detected in samples receiving up to the 0.02% IGF-I ($p < 0.001$), after which a decrease in GAG content was observed in 0.07% and 0.7% IGF-I samples when compared with the 0.02% IGF-I group (Figure 25A). In contrast, GAG density remained relatively unchanged at each time point. However, samples receiving 0.02% IGF-I exhibited higher GAG density than other groups after 8 weeks (Figure 25B).

Samples were stained with safranin-O to analyze the proteoglycan distribution across the tissue. At 2 weeks, samples receiving IGF-I through exogenous delivery (Figure 26A) or controlled release (Figure 26B) exhibit homogenous staining for proteoglycans across the tissue sections. Samples that did not receive IGF-I had strong

safranin-O staining, but this staining was interspersed (Figure 26C). After 8 weeks, samples receiving IGF-I by either means continued to display homogenous staining for proteoglycans, but slight staining on the microspheres (Figure 26D, E). However, samples without IGF-I continued to demonstrate interspersed and disconnected tissue staining patterns (Figure 26F).

Detection of collagen type II accumulation suggested a dose response to IGF-I at each time point, with the most defined dose response at 8 weeks. After 2 or 4 weeks, samples receiving 0.02% or 0.07% IGF-I presented the highest collagen levels. However, at the 8 week time point, a shift in the dose response to IGF-I was observed. Samples increased in collagen type II content with the highest content exhibited in 0.02% IGF-I samples, but the collagen type II content decreased at higher doses of 0.07% and 0.7% (Figure 27A). In addition, collagen content remained relatively unchanged after 2 or 4 weeks, but exhibited increased collagen densities in all groups after 8 weeks, with samples receiving 0.02% IGF-I demonstrating the highest collagen density (Figure 27B).

Cartilage sections were immunostained for collagen type I or collagen type II. Samples immunodetected for collagen type II exhibited homogenous positive staining, indicated by the brown coloration (Figure 28A, C). Samples receiving IGF-I through controlled or exogenous delivery exhibited negative staining for collagen type I (Figure 28B), while samples that did not receive IGF-I exhibited slight areas of positive staining for collagen type I (Figure 28D).

Immunostaining was also used to detect the presence of IGFBPs in engineered cartilage. Immunostaining in samples receiving IGF-I suggested the presence of both

IGFBP-2 (Figure 29A) and IGFBP-6 (Figure 29B), which was indicated by a brown coloration across the tissue sections. In contrast, samples that were not delivered IGF-I exhibited a few positive areas for IGFBP-2 (Figure 29C) and IGFBP-6 (Figure 29D) that were interspersed with larger areas staining negative for both binding proteins, indicated by the purple counterstain.

Discussion

PLG microspheres have been used in tissue engineering for the controlled release of growth factors, but have not been utilized in vitro as a simultaneous controlled delivery vehicle and tissue engineering scaffold. This study demonstrates the use of PLG microspheres as both a scaffold and controlled delivery vehicle of growth factor (IGF-I) for in vitro cartilage regeneration. In addition, this study shows controlled release of IGF-I from PLG microspheres dose-dependently enhances cartilage matrix regeneration.

Because of its well-known stimulatory effects on cartilage matrix synthesis,^{56,58,60} various delivery methods for adding IGF-I to tissue engineering designs exist.^{24,27,70} However, many of these delivery methods are arduous, as they require repetitive addition of growth factor in media changes or manipulation of the chondrocyte by transfection or transduction. Thus, controlled release of growth factor from a scaffold that can simultaneously support cartilage growth would be desirable. To identify whether controlled release of IGF-I provided a substitute delivery method, we compared developing cartilage that received IGF-I via controlled release, exogenous IGF-I addition in media, and no IGF-I. After 8 weeks, all samples receiving IGF-I formed more

cartilage tissue and accumulated matrix to a greater extent than samples without IGF-I. In addition, controlled release of IGF-I was as effective as exogenous delivery in forming tissue and generating matrix. Overall, two differences were detected between groups with different delivery methods. The first variation was noted in GAG accumulation at 2 and 4 weeks, as samples receiving exogenous delivery significantly amassed more GAG ($p < 0.001$). Similarly, previous results (see chapter IV) have demonstrated slower GAG accumulation at these early time points, with increased accumulation after 8 weeks. The increased GAG content after 8 weeks seems to correlate with microsphere degradation and increased IGF-I release. The second difference indicated a more perplexing variation with regard to significantly higher tissue mass in samples receiving exogenous IGF-I delivery after 8 weeks ($p < 0.001$). At this same time point both groups exhibited equal accumulation of proteoglycans and collagen, and the differences seen may be attributed to differences in water content. However, dry weights would need to be evaluated to confirm this speculation.

Given that controlled release enhanced matrix accumulation and cell content to the same extent as exogenous delivery, we then wanted to determine the range of effective doses of controlled release in this system for in vitro cartilage regeneration. Doses between 0-0.7% IGF-I were evaluated, containing total protein loads of up to 48 μg of IGF-I. Over 8 weeks, between 65 and 100% of the IGF-I was released, and up to 32 μg of protein was delivered. Had these doses been delivered consistently, this would correlate to up to 333 ng/ml over 8 weeks, which can be related to typical doses of 10-300 ng/ml used for exogenous addition, direct injection, or encapsulation of IGF-

I.^{24,27,32,61} However, in a controlled delivery system, protein release is dependent on microsphere degradation and therefore not consistent over time,^{44,46,47} as indicated in this study with the highest concentration of IGF-I being released between weeks 4 and 6.

Dose dependent trends exhibited in matrix accumulation appeared to adhere to release patterns. At all time points, dose-dependent increases in GAG accumulation were exhibited, while dose-dependent increases in collagen content was only observed after 8 weeks. However, dose-dependent trends for both collagen and GAG contents were most pronounced at 8 weeks, which would coincide with the samples receiving the highest release of IGF-I. In addition, increases in GAG and collagen content followed similar dose-dependent curves; GAG and collagen levels increased dose-dependently in groups receiving up to 0.02% IGF-I, while at doses higher than 0.02% IGF-I GAG and collagen contents decreased. Collectively, these data suggest that the most effective doses for cartilage regeneration are different from those doses found to be stimulatory when added by exogenous addition or direct injection. Moreover, it has been suggested that in vitro delivery of supraphysiologic doses of IGF-I do not have a negative effect on chondrocytes or matrix production,⁶⁸ but in this system, the highest doses of IGF-I (0.07% or 0.7% IGF-I) did not produce as much matrix as other doses, suggesting negative effects on matrix production.

The presence of IGFBPs suggests one possible explanation for decreased matrix content at higher doses. IGFBPs are known to attach to components within the matrix¹⁰⁷⁻¹⁰⁹ or directly to cell surfaces¹¹⁰ and specifically, in cartilage tissue IGFBP-2 localizes around the membrane of the chondrocyte,¹¹¹ while IGFBP-6 likely binds to

matrix materials located further from cells and blocks diffusion of IGF-I.⁹¹ Thus, although IGFBP-2 is found in these engineered tissues, the presence of IGFBP-6 may contribute to increased binding of IGF away from the cell. In this study qualitative assays were used to demonstrate the presence of IGFBPs in engineered cartilage using the controlled release system. However, it would be interesting to quantitatively determine the concentrations of specific IGFBPs relative to other IGFBPs in the tissue to further understand the paradigm of binding protein location and influences on IGF-I interaction with chondrocytes.

In addition to IGFBP location, another explanation for the plateau in matrix molecule content after 8 weeks in the higher IGF-I doses could be that IGF-I itself is regulates the production of additional IGFBPs. Work has indicated that stimulation of chondrocytes with IGF-I increases IGFBP content.^{105,106} Although the scope of this work did not specifically seek to evaluate delivery concentrations of IGF-I that would stimulate IGFBP production, the understanding gained from this study suggests that as microsphere degradation ensues and release of IGF-I increases, doses of 0.07% IGF-I and higher likely increase IGFBP concentrations. The supposed increase in IGFBP concentrations likely inhibits IGF-I from interacting with its receptor to stimulate increases in GAG and collagen type II content.

Overall, this study demonstrates effective production of engineered cartilage using PLG microspheres as both a growth factor delivery system and polymer scaffold, and controlled release of IGF-I offers an alternative method to exogenous protein delivery, as both delivery methods stimulated matrix accumulation to the same extent.

Moreover, evaluation of cartilage tissue regeneration suggested that 0.02% IGF-I provides the most effective dose for cartilage tissue regeneration.

IGF Dose	Total IGF in system (ug)	ng IGF-I/ml (8 wk)
0%	0	0
0.0007%	0.112	1.15
0.007%	1.12	11.7
0.02%	3.2	33
0.07%	11.2	117
0.7%	112	1117

Table 7. Total IGF-I protein loaded for each dose condition. PLG microspheres with a 1% IGF-I load were fabricated using the ProLease® process. Loaded and unloaded microspheres were mixed in different ratios to obtain a range of IGF-I effective loads. Total IGF-I protein in the 16 mg of PLG microspheres is reported for each effective dose, and the total amounts of IGF-I released for each dose was normalized to total media used over 8 weeks (96 ml media).

IGF-I dose	Total IGF-I (ug) released after 8 weeks	# of Days for 50% Release
0%	0	-
0.0007%	0.2	29
0.007%	0.771	36.5
0.02%	2.37	34
0.07%	7.27	38
0.3%	32.3	37.5

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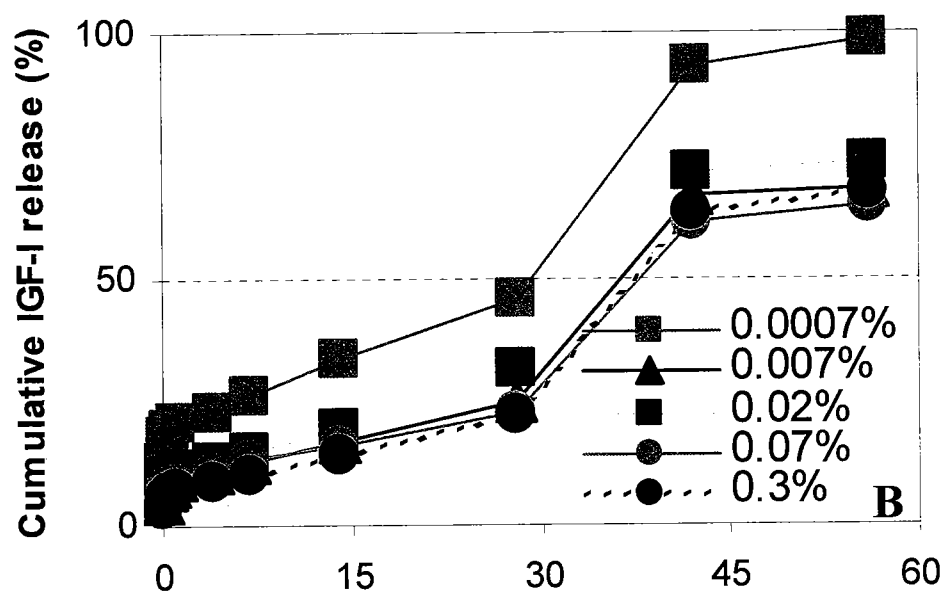


Figure 22. Controlled release of IGF-I from PLG microspheres. PLG microspheres with a range of IGF-I effective loads were incubated in F-12 media. At time points between 0 and 56 days, media was removed and replaced with fresh media. IGF-I content in the media was evaluated using an IGF-I ELISA, as described in materials and methods. A. Total protein release from microspheres containing different concentrations of IGF-I was evaluated out to 8 weeks. Microspheres released between 0.2 and 32 μg of IGF-I depending on the IGF-I dose, with all groups releasing 50% of the protein load around 30-40 days. B. Cumulative IGF-I release from different microsphere groups indicated that around 70% of the total IGF-I in the system was released by 8 weeks for IGF-I doses ranging between 0.007% and 0.3%, whereas microsphere doses of 0.0007% released almost 100% of their contents.

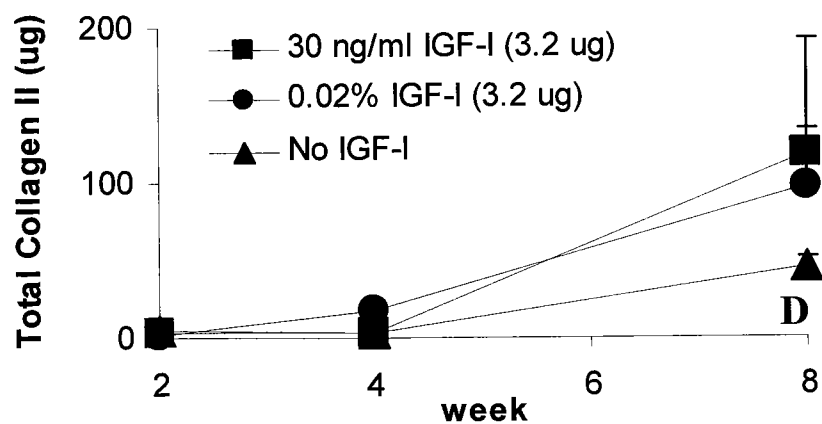
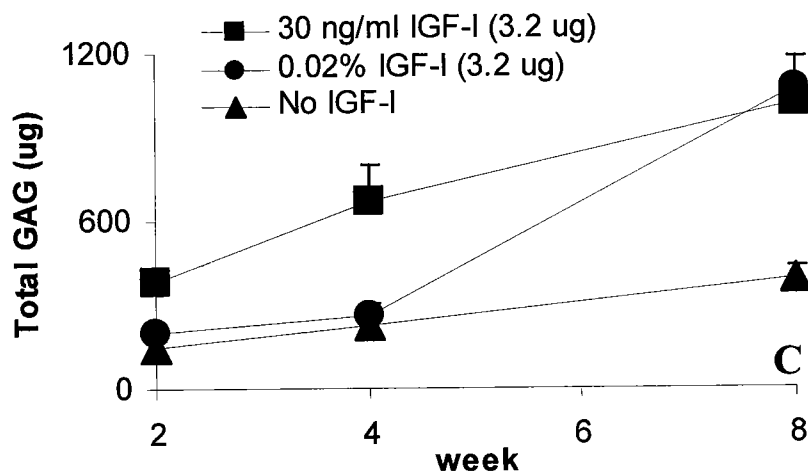
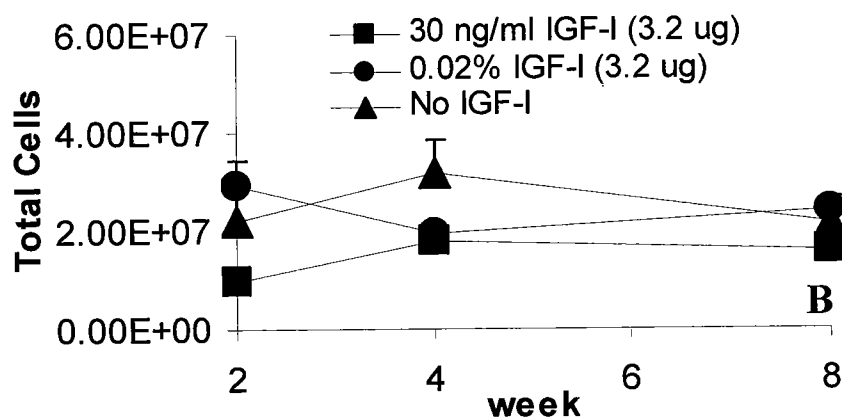
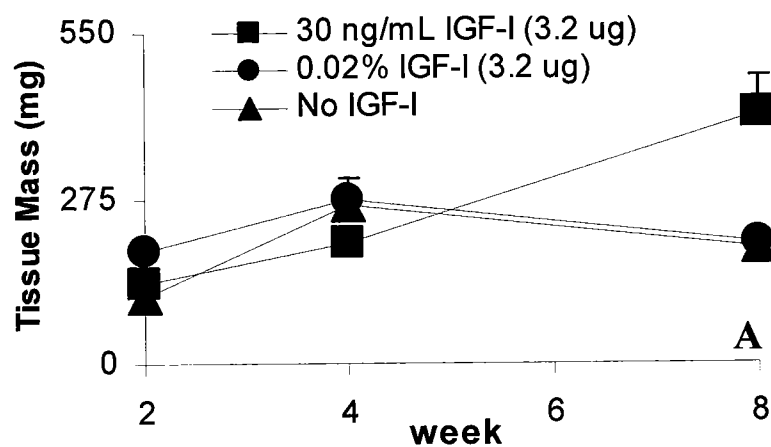


Figure 23. Controlled delivery of IGF-I offers an alternative to exogenously delivered IGF-I in cartilage tissue engineering. Cartilage formation was assessed in samples receiving IGF-I by exogenous delivery, controlled release of IGF-I, or no IGF-I delivery with respect to tissue mass (A), cell number (B), GAG content (C) and collagen type II accumulation (D). Tissue mass was increased after 8 weeks only in samples receiving exogenous IGF-I (A), but by 8 weeks delivery of samples formed using controlled IGF-I release accumulated GAG and collagen type II to the same extent as samples receiving exogenous IGF-I (C,D). Samples from both exogenous delivery and controlled release groups formed significantly more GAG and collagen than cartilage that did not receive any IGF-I ($p < 0.001$). No differences were detected in cell number between any of the groups (B). $n=3$ for exogenous IGF-I samples, and $n=6$ for IGF-I controlled release samples and for samples without IGF-I.

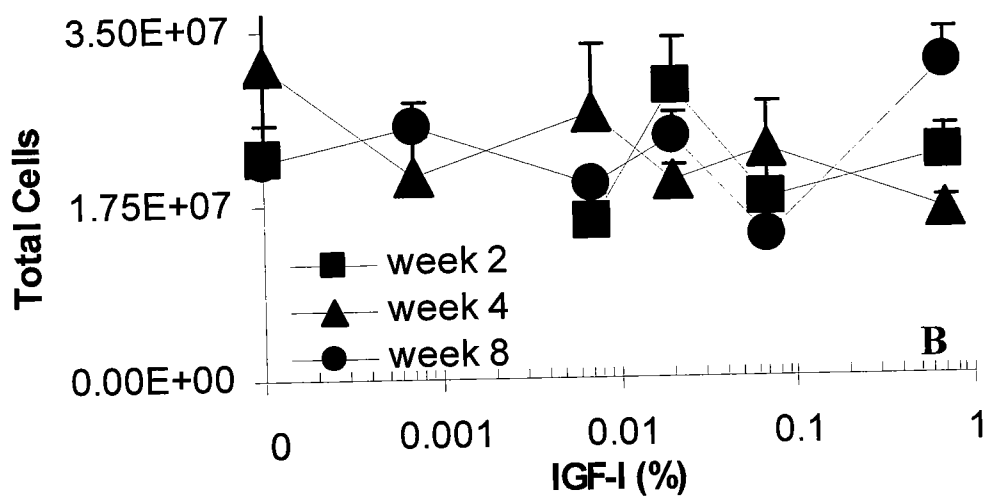
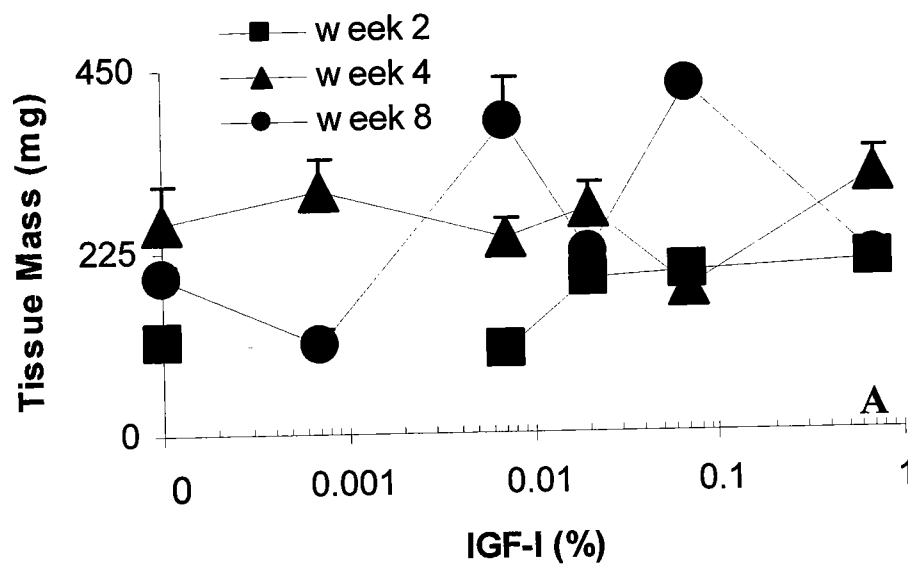


Figure 24. Controlled release of IGF-I on tissue mass and cell number is not dose-dependent in vitro. Controlled release of IGF-I on cartilage formation was assessed over a range of doses (0-0.07% IGF-I). The effect of IGF-I concentration was evaluated for cartilage tissue mass (A) and cell number (B). No differences in tissue mass at 2 or 4 weeks or in cell number at any time point were observed. IGF-I significantly increased tissue mass in samples receiving 0.007% or 0.07% IGF-I ($p < 0.001$) after 8 weeks, but IGF-I did not induce a dose-dependent increase in tissue mass at this time point.

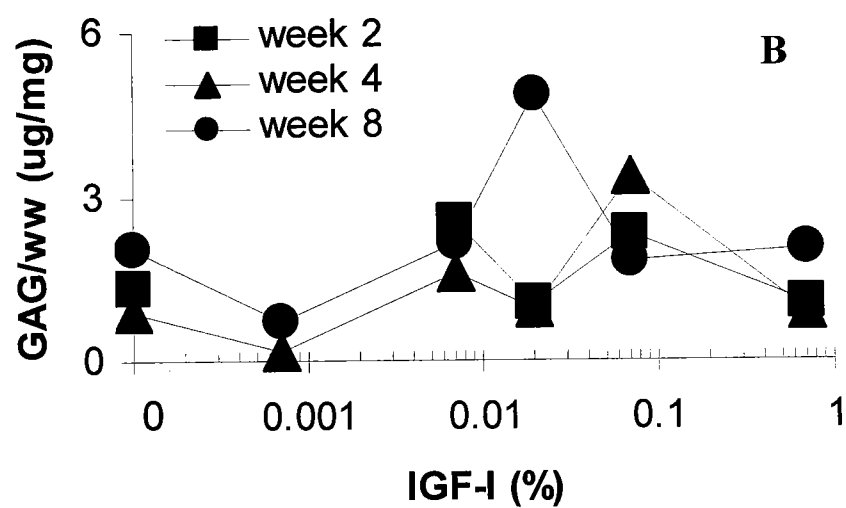
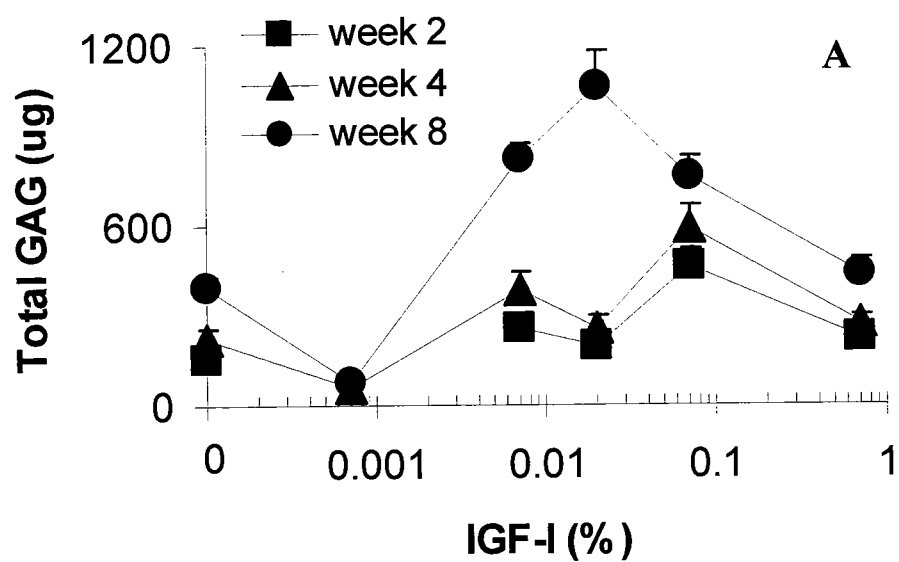


Figure 25. Dose-dependent effects of IGF-I were observed in GAG accumulation. A. IGF-I induced dose-dependent increases on GAG accumulation at each time point. After 2 or 4 weeks, samples with effective loads of 0.07% accumulated the most GAG. By 8 weeks, a shift in the dose-response curve was observed, where samples receiving 0.02% IGF-I had the highest GAG content ($p < 0.002$). Samples receiving doses higher than 0.02% IGF-I indicated decreased GAG content at 8 weeks ($p < 0.001$). B. GAG content was normalized to tissue wet weight, and similar dose-response curves were observed for GAG density. Samples receiving 0.07% IGF-I exhibited the highest GAG density after 2 or 4 weeks, but at the 8 week time point, samples formed with 0.02% IGF-I effective loads exhibited the highest GAG density. $n=6$ for all groups.

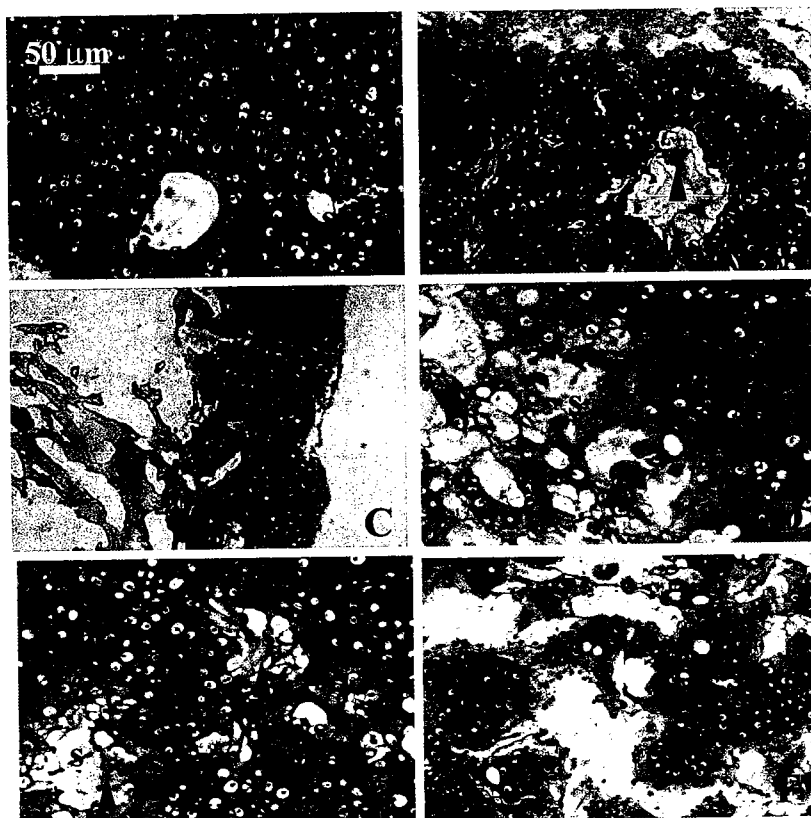


Figure 26. Cartilage sections from 2 week samples (A-C) and 8 weeks samples (D-F) were stained with safranin-O as an indicator of proteoglycan deposition throughout the matrix. The presence of microspheres, indicated by (s), was clearly detectable in samples that received exogenous IGF-I (A, D) or controlled release of IGF-I (B, E), but microspheres were difficult to decipher in sections from cartilage tissue formed without IGF-I. In samples receiving IGF-I, matrix deposition appeared homogenous across the sections as more matrix was deposited onto microspheres (indicated by black arrowheads). In contrast, at all time points, samples that did not receive IGF-I exhibited a lack of tissue continuity and sparse matrix deposition. All samples were magnified 200x.

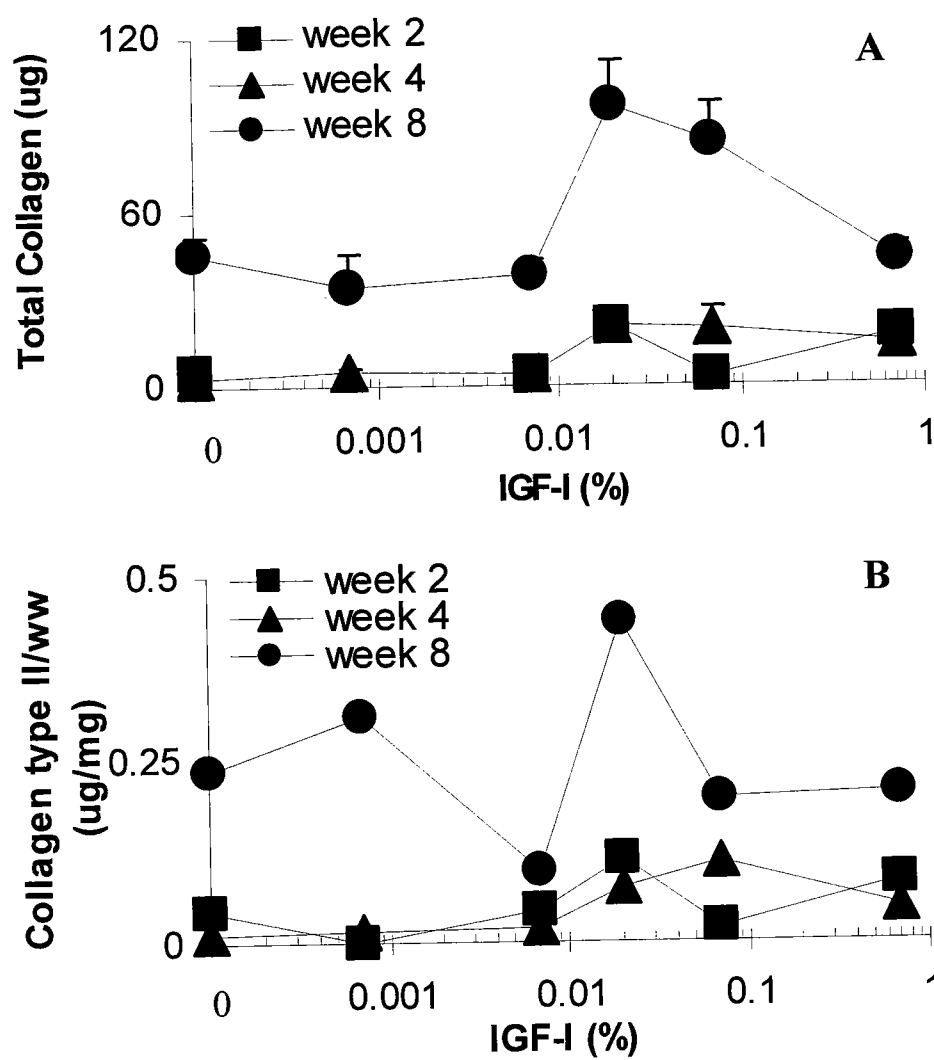


Figure 27. IGF-I induced dose-dependent increases in collagen type II levels. Collagen type II content was evaluated by ELISA, as described in materials and methods.

Collagen type II content was evaluated as total collagen type II (A) or normalized to tissue wet weight to obtain collagen type II density (B). A. Collagen content remained low at 2 and 4 weeks, with slight dose-responses to IGF-I at these time points. After 8 weeks, total collagen type II levels followed a more pronounced dose-dependent increase to IGF-I. Increases in collagen content were observed in samples receiving up to 0.02% IGF-I, after which a decline in total collagen was detected. B. Analysis of collagen density revealed slight dose-responses to IGF-I at weeks 2 or 4. A more pronounced dose-response curve was observed after 8 weeks, with the highest collagen density exhibited in samples receiving 0.02% IGF-I. Although trends were observed, no statistical differences were detected. n=3 for all groups.

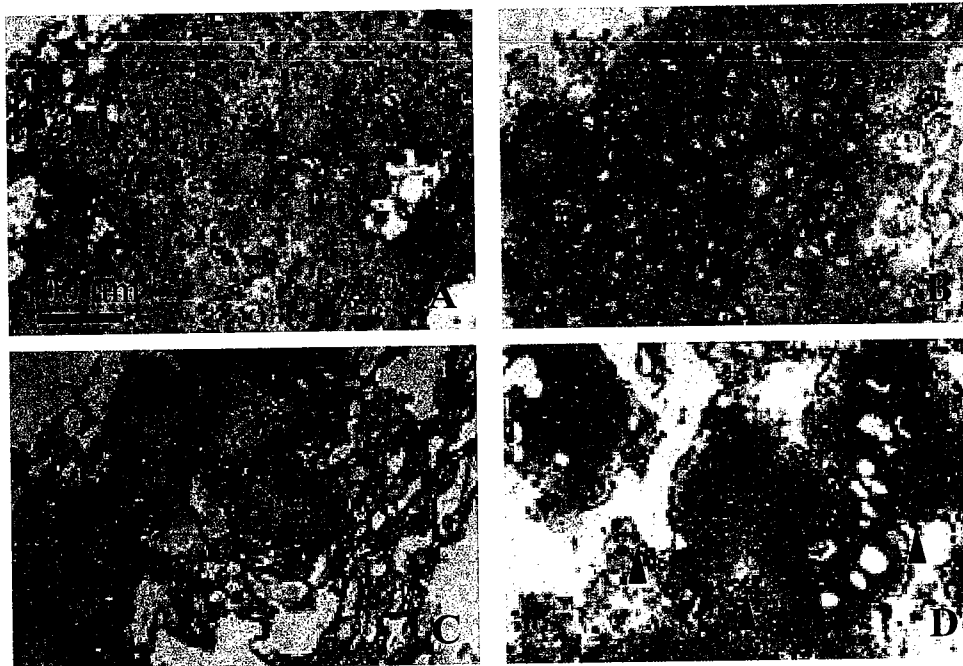


Figure 28. Samples receiving IGF-I maintain the collagen type II phenotype.

Immunostaining for collagen type II (A, C) and collagen type I (B, D) revealed differences for tissue samples with IGF-I (A, B) or without IGF-I (C, D). All samples retained their native collagen type II phenotype, as indicated by the uniform brown staining throughout the sections (A, C). Samples receiving IGF-I displayed negative staining for collagen type I as indicated by the homogenous blue staining of the tissue (B). In contrast, cartilage samples formed without IGF-I exhibited some positive brown staining for collagen type I, indicated by the black arrowheads (D). All pictures are magnified 100x.

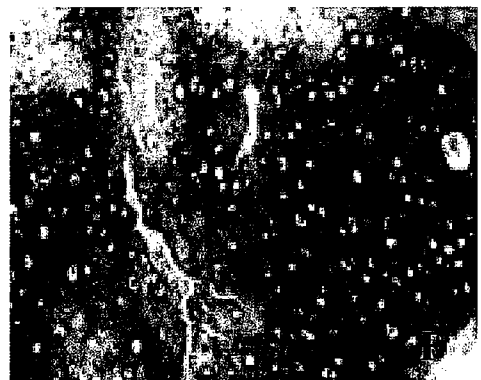
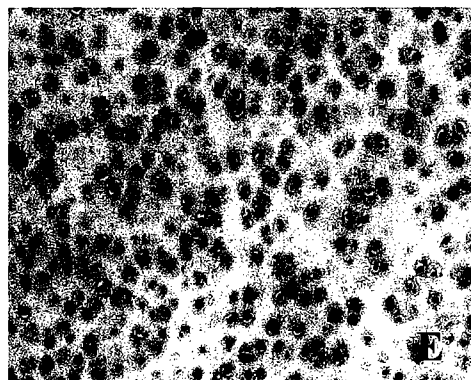
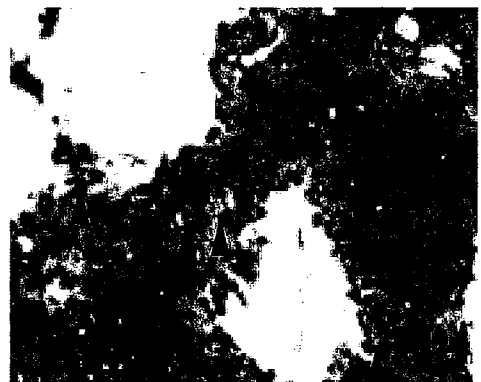
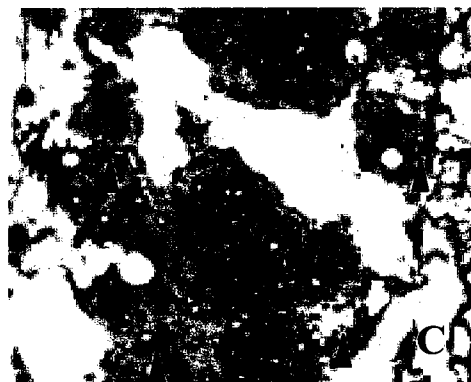
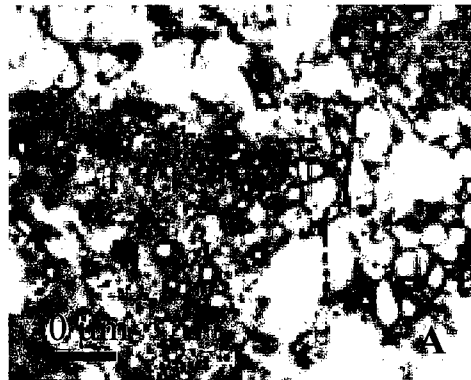


Figure 29. IGFBPs are present in tissue engineered cartilage. Immunostaining of 8 weeks sections from samples with (A, B) or without (C, D) IGF-I indicated differences in staining patterns for IGFBP-2 (A, C) and IGFBP-6 (B, D). All samples receiving IGF-I were positive for both IGFBP-2 and IGFBP-6, as indicated by the brown color. In contrast, IGFBP-2 and IGFBP-6 were less evident in samples that did not receive IGF-I; fewer areas of positive staining (indicated by brown and black arrowheads) were juxtaposed with large areas of negative staining (indicated by purple) for IGFBP-2 and IGFBP-6. Native cartilage was immunostained for IGFBP-2 (E) or IGFBP-6 (data not shown) were similar in staining patterns, with positive staining localized around the chondrocytes. Panel F depicts a typical engineered cartilage section immunostained without primary antibodies to IGFBP-2 or IGFBP-6, which only exhibited evidence of the negative hematoxylin (purple) counterstain. All pictures were magnified 200x.

CHAPTER VI

DISCUSSION

The polymer scaffold offers an integral component to the tissue engineering design, and therefore provides a unique opportunity to control the process of tissue generation. Polymers used as tissue engineering scaffold typically present a range of parameters such as strength, durability, or biocompatibility, but can also present shortcomings such as lack of control of polymer degradation or little to no support of cellular attachment. Although much work has been done to circumvent such limitations, little work has been done to investigate the impact of the polymer or the scaffold as an active component in cartilage tissue engineering. This discussion will point to the development and relevance of a novel PLG microsphere system, used as a simultaneous polymer scaffold and growth factor delivery vehicle, to understand the role of the polymer scaffold in chondrogenesis. Data presented in this body of work suggests that the polymer and scaffold can be manipulated to incorporate changes that support and enhance cartilage tissue engineering. Specifically, it was found that polymer and scaffold changes that slowed microsphere degradation or gradually released IGF-I enhanced cartilage formation.

The Presence of a Polymer Scaffold in Chondrogenesis

Many studies in tissue engineering have recognized the value in using a polymer scaffold for cell attachment and the support of tissue development. In this study, we have compared cartilage tissue formed from cells alone or from cells and microspheres, and overall we have observed more tissue and matrix formation in samples prepared with microspheres. Moreover, our data suggested that microspheres preserve space for later tissue deposition, purportedly by allowing chondrocytes to migrate into the space occupied by degrading microspheres to deposit additional matrix. This is in contrast to tissue formed from cells alone, which exhibit abnormal cell spacing as evidenced by this study and others when cartilage is regenerated with only chondrocytes.^{17,19}

In this way, the scaffold may also serve in the generation of cartilage with higher GAG content. Cell spacing can influence multiple tissue characteristics, including the cartilage matrix. The ECM of cartilage consists of the pericellular matrix, which directly surrounds the cell, an intermediary matrix termed the territorial matrix, and an interterritorial matrix that is further removed from the cells.² Thus, it is conceivable that in tissue exhibiting hypercellularity and a lack of appropriate cell spacing, the overall amount of pericellular matrix would increase. In turn, multiple scenarios could be construed. The pericellular matrix is known to contain large amounts of type VI collagen and proteoglycans, while the interterritorial matrix provides the bulk of cartilage tissue, principally composed of collagen type II and aggrecan.² A shift in these tissue constituents may result in mechanically inferior tissue. Similarly, increases in pericellular matrix content may also have effects on matrix remodeling. Recently,

ADAMTS4 (a disintegrin and metalloproteinase with thrombospondin-like motif) has been shown to become activated on the cell surface.¹¹² ADAMTS4 degrades aggregating proteoglycans,¹¹³⁻¹¹⁵ and as such an increased pericellular matrix would provide a larger proteoglycan pool for susceptibility to matrix remodeling.

In addition to concerns of tissue inconsistencies with native cartilage, the presence of a polymer scaffold may enhance tissue formation by minimizing oxygen conditions. Detection of the diffusion of molecules such as oxygen demonstrated a gradient in the tissue-engineered construct, with the lowest oxygen levels at the center of the tissue¹¹⁶. Low oxygen levels have been shown to induce redifferentiation of dedifferentiated chondrocytes.^{84,85} In addition, high oxygen tensions have been shown to increase GAG content initially, but low oxygen tensions significantly increased GAG content over time when compared to samples with elevated oxygen levels.⁸⁵ In this study, all cartilage samples formed with or without microspheres exhibited similar GAG contents after 2 weeks. However, cartilage formed with cells and microspheres exhibited an overall higher GAG content at later time points. Taken together this data suggests that limitations in oxygen diffusion due to the presence of microspheres, which generate greater tissue mass and subsequent diffusional distances, may support and generate better matrix accumulation.

Changes in Polymer and Scaffold Affect Chondrogenesis

The effect of polymer degradation has been an important topic for consideration in tissue engineering studies because of potential toxicity of acidic degradation

products¹¹⁷ and harmful effects on cells.⁵⁰ Therefore, we evaluated polymer and scaffold parameters that would slow degradation rate and production of polymer breakdown in the form of carboxylic acid end fragments. One parameter that we evaluated was microsphere size, Increasing microsphere size would also provide additional surface area for cell attachment and degrade slower because they require more hydrolytic events leading to polymer breakdown and degradation. We found that the size of the microsphere did not change the amount of matrix accumulated or number of cells that proliferated. Rather, weak staining for proteoglycans around the larger microspheres was detected. It is likely that the weak staining for proteoglycans can be attributed to low pH since drops in pH do not increase matrix production.⁵⁰ Moreover, larger microsphere likely retained acidic fragments longer because the diffusional distance for clearance of these fragments was greater than in microspheres with smaller diameters. Thus, the build-up of these fragments leads to a lower pH.¹¹⁸ It is likely that when the microspheres had bioeroded enough to increase pore size, the acidic fragments diffused out of the microspheres in large quantities, which likely had toxic effects on the production of matrix from the surrounding cells.

Work to control polymer degradation has often included the addition of molecules that would buffer the acidity of the degrading environment.²³ However, negative affects on matrix production were observed in samples that received MgOH_2 buffer. It is likely that large amounts of released buffer led to decreased matrix accumulation, which could be confirmed by evaluating the release profiles for buffered microspheres, but was not undertaken in this study. Further, it is unclear whether the buffer induced the cells to

apoptose or necrose. If the buffer caused the cells to apoptose, it would be important to understand the potential signaling molecules that are upregulated or activated under these conditions since apoptosis can be induced by numerous molecules within the signaling cascade.¹¹⁹

The addition of a hydrophobic cap provides another means to slowing polymer degradation and limiting the generation of high concentrations of acidic ends. Polymer capping decreases the rate at which water is absorbed into the polymer²³ and generates fewer acidic ends. In this study, tissue formed from capped polymer exhibited increases in matrix accumulation and were not inhibited by the generation of acidic fragments, likely because acidic by-products were able to diffuse out of the microspheres faster than they were produced. Capped microspheres were designed with polymer that has roughly the same degradation time as the 50 kDa polymer. While both of these polymers showed slowed degradation, the addition of a capped end group also increased the hydrophobicity of the polymer and microsphere, which may preclude cell attachment⁷⁹ and limit matrix deposition on these microspheres.

Degradation can also be altered by increasing the molecular weight of the polymer used to form the microsphere. High molecular weight polymers require an increased number of catalytic events to break down the polymer, fewer acidic by-products are generated, and the polymer degrades slower than lower molecular weight polymers.²³ In this study, microspheres formed with 50 kDa polymer led to cartilage tissue with enhanced matrix production and evidence of matrix deposition onto the microspheres after 8 weeks. Although microspheres made with 50 kDa polymer

supported cell attachment and tissue growth seemingly better than other microspheres used in this study, only two different molecular weight polymers were analyzed. Thus, polymers with a range of molecular weights would provide a better understanding of the role that molecular weight plays in cartilage formation.

It is clear from these studies that controlling polymer degradation is a significant issue in cartilage tissue engineering. However, assessment of the individual factors that may affect degradation give an understanding of how specific characteristics, such as chemistry, size, molecular weight, or buffer, affect cartilage development. This study marks the beginning of the evaluation of such parameters, but there are many more scaffold features that could influence tissue development, including pore size and porosity of the scaffold, that would need to be assessed. The PLG microspheres used for tissue engineering offer a plausible system for evaluation of such parameters since alterations in the polymer or scaffold resulted in similar behavior both in vivo and in vitro, suggesting that the observed effects can be attributed to the specific modified parameter.

Chondrocyte Metabolism

Organization of the cartilaginous matrix incorporates multiple proteins and proteoglycans in an interconnected network, and the sole responsibility of the chondrocyte is to synthesize and maintain this network. The importance of an organized and interconnected ECM is evidenced in disease conditions such as osteoarthritis that presents with joint degeneration and release of proteoglycan and collagen

molecules.¹²⁰⁻¹²² Subsequent loss or low concentrations of these molecules leads to insufficient tissue mechanics and reduced compressive and/or tensile moduli.¹²³⁻¹²⁶ Thus, to determine the viability of a novel tissue engineering strategy, the analysis of matrix content and maintenance of the chondrocytic phenotype is critical.

In this study, a novel cartilage repair strategy using a PLG microsphere scaffold, with the potential for controlled delivery of growth factor, has been evaluated with respect to matrix development both in vivo and in vitro. Native cartilage tissue exhibited on average 4-5% GAG per tissue wet weight and 0.1-10% collagen type II/ tissue wet weight.^{26,37,127,128} In addition, engineered cartilage formed under varying conditions has demonstrated a range of GAG densities from 1-5% of tissue wet weight and collagen densities ranging from 0.05-4% of tissue wet weight over a duration of 2 to 30 weeks.^{26,31,32,37,67,127,128} In the current studies, up to 3.5% GAG density and 0.3% collagen density has been generated in vivo, while in vitro cultured cartilage displayed up to 2.2% GAG density and 0.04% collagen density. Overall, our methods produced levels of collagen and GAG that are consistent with other engineered cartilage models, serving to validate the cartilage tissue engineering strategy of a simultaneous scaffold and growth factor delivery vehicle.

Although the aforementioned results lend promise to the utility of this novel cartilage repair method, slight shortcomings in matrix accumulation were observed in vitro in cartilage samples receiving IGF-I; specifically, low collagen type II accumulation was exhibited in all samples regardless of IGF-I dose. Collagen synthesis and protein assembly is a complex process, preceded by transcription of the Col2a1 gene. Upon

production of the collagen molecule, intracellular, self-organization of three α_1 collagen molecules forms a triple helical molecule with telopeptide regions that require cleavage prior to ECM incorporation.¹²⁹ Although, IGF-I has been demonstrated to upregulate both collagen mRNA and protein levels,^{24,130} it is unclear whether the lack of collagen accumulation can be attributed to changes in chondrocyte metabolism at the level of transcription or translation, improper collagen assembly, or protein degradation by collagen specific proteases, such as matrix metalloproteases.¹³¹

Although IGF-I is a potent cartilage matrix stimulator, matrix accumulation in vivo is a collective effort of many growth factors. In addition to IGF-I, both TGF- β and bFGF have been shown to stimulate matrix production.^{32,60,67} Moreover, some tissue engineering strategies have taken advantage of using simultaneous or sequential delivery of multiple growth factors to promote increases in matrix parameters. When TGF- β and IGF-I were used in combination, GAG density was increased as compared to samples receiving just IGF-I or just TGF- β .²⁵ Similarly, chondrocytes expanded with FGF-2 and subsequent 3-dimensional culture with bone morphogenetic protein-2 maintained chondrocyte phenotype and exhibited increased matrix accumulation.¹³² Using a PLG microsphere scaffold would facilitate simultaneous and/or sequential growth factor delivery for cartilage tissue engineering. Microspheres can be loaded with different growth factors and mixed to achieve desired growth factor ratios and release. Chondrocytes seeded with mixed microsphere populations could receive growth factors at equal rates of release, or, by varying microsphere parameters, one growth factor could be released to the chondrocytes prior to release of the second.

Alternative Methods to Exogenous IGF-I Delivery

The importance of IGF-I in enhancing chondrogenesis is indicated by the multiple systems designed to incorporate IGF-I into tissue engineering. The addition of exogenous growth factor^{24,32} is limited by protein half-life,⁹⁰ and as such, alternative delivery methods have been developed. In this study we have implemented sustained and controlled delivery of IGF-I during neo-cartilage formation. Used in this cartilage tissue engineering strategy, we demonstrated that controlled delivery of IGF-I offers a viable alternative to exogenous growth factor delivery.

Other methods have also been developed to counter limitations on protein half-life, including gene therapy applications. The amalgamation of tissue engineering and gene therapy, in its relatively young stage, has been attempted via transfection of primary articular chondrocytes with a plasmid containing the IGF-I gene for use in regenerating cartilage tissue.^{69,70} Transfected chondrocytes transplanted onto articular cartilage explants formed a layer of tissue on the explant with enhanced GAG synthesis.⁷⁰ In addition, increases in GAG stimulation, collagen type II content, and the confined-compression equilibrium modulus were evident after 4 weeks in cartilage engineered with IGF-I transfected chondrocytes embedded in PGA scaffolds.

Similarly, ideas for joining gene therapy with tissue engineering have been approached using naked plasmid DNA embedded in polymer. Current studies have depended on the migration of cells into the DNA-laden polymer matrix, relying the cells themselves to ingest the DNA plasmid, a method that has been shown to repair bone

defects.¹³³ This model likely has applications for polymer matrices pre-seeded with both DNA and cells, which would potentiate more efficient delivery of DNA and transfection of the cells given the close proximity of the two constituents. In addition, controlled DNA delivery from PLG microspheres has also been demonstrated in vitro,^{134,135} but as of yet has not been used to generate engineered tissue. Overall, however, the utility of genetic manipulation will be understood by future experiments comparing its efficiency with controlled and sustained protein delivery.

Effective Dose of IGF-I Differ in Vivo and In Vitro

Given the multitude of literature demonstrating the stimulatory effects of the growth factor, it was expected and confirmed that controlled delivery of IGF-I enhanced chondrogenesis. A first step in validating this method for tissue engineering applications is to evaluate a range of IGF-I doses effective in a controlled release system for relevance both in vivo and in vitro.

The fact that the effective dose of IGF-I varies in vivo and in vitro raises numerous questions about the actions of IGF-I. The most obvious question raised concerns the discrepancy in effects on matrix production in doses higher than 0.02% IGF-I. Such inconsistencies could be explained by the diffusion of released IGF-I out of the developing tissue nodule and into the host tissue in vivo, such that all IGF-I delivered by controlled release would not act on the developing cartilage tissue. Consistent with this theory, when PLG microspheres containing GH were locally implanted in rhesus monkeys, an increase in serum GH levels, IGF-I and IGFBP-3 levels were observed.⁴³

Thus, it would be important to evaluate the surrounding endogenous tissue for detection of IGF-I actions. Further, one might expect that more regular media changes would ensure that IGF-I did not build up in vitro, enabling any IGF-I that diffused out of the tissue to enter back into the construct.

A second question surrounds the role and production of IGFBPs in the engineered tissue. Because IGF-I has been shown to increase the production of IGFBPs^{105,106} and in these studies we have only qualitatively demonstrated the presence of IGFBP-2 and IGFBP-6 in vitro, quantification of these binding proteins would provide an initial understanding to the role that IGFBPs in cartilage tissue engineering. Specifically, comparing the relative concentrations of binding proteins in samples receiving different doses of IGF-I would enable an understanding as to whether IGFBPs inhibited IGF-I actions at the highest doses in vitro.

An equally plausible explanation for differences in matrix production in samples generated with the highest IGF-I doses both in vivo and in vitro could be the regulation of IGFBPs themselves. Proteolysis of IGFBPs has been demonstrated in most IGFBP family members, which can be attributed to multiple enzyme classes including serine proteases, matrix metalloproteases and cathepsins.¹³⁶ There is evidence suggesting that in some instances, cleavage of the IGFBP releases intact IGF-I from the complex.¹³⁷ As these proteolytic enzymes are likely more abundant in vivo because of the additional supply from the host, a balance of complex cleavage and IGF-I release may be facilitated in vivo allowing the high doses of IGF-I to be effective.

Clinical Relevance

This study demonstrates the feasibility of using a simultaneous growth factor delivery system and scaffold to support cartilage tissue growth. While the support of tissue growth must be demonstrated, this study did not assess the use of this method for cartilage repair, the ultimate goal of tissue engineering. Currently, the only clinically approved procedure for cartilage repair is autologous chondrocyte transplantation, involving the expansion and delivery of autologous chondrocytes to the defect and securing the cells with a periosteal graft.^{138,139} This cell-based repair technology has demonstrated variable results, and has shown the development of fibrocartilage in some cases.^{140,141} Although this is the only clinically approved method for cartilage repair, the literature expounds upon numerous applications for repair using tissue engineering methods with both chondrocytes and polymer scaffolds.

The expression of fibrocartilage can lead to mechanical instability, and therefore, it is crucial that a clinically relevant cartilage tissue engineering method produces tissue with solid mechanical properties. One of the shortcomings of the work presented in these studies is a lack of understanding of such mechanical properties that would give an indication of the tissue's ability to distribute stress and bear weight. Because typical increases in GAG and collagen matrix have also correlated to substantial increases in various modulus parameters of the tissue,^{31,32,164} we would expect that the increases in GAG and collagen in these studies would translate into higher compressive or tensile moduli in cartilage formed with 50 kDa polymer or optimal doses of IGF-I. However, the clinical relevance and comparison of engineered cartilage to the compressive and

tensile properties of native cartilage tissue necessitate mechanical testing of cartilage formed using PLG microspheres.

In addition to the indication of tissue function by mechanical assessment, cartilage structure and organization also warrants characterization for a valid cartilage tissue engineering model. Cartilage tissue is a multi-layered tissue with superficial, middle and deep zones. Chondrocytes within these different zones exhibit different metabolic activities that maintain mechanical functions specific to that zone. For example, chondrocytes of the superficial zone, the region specialized for stress distribution and joint lubrication, secrete GAG at relatively low rates^{142,143} and produce lubrication proteins such as superficial zone protein.¹⁴⁴ Deeper layers exhibit increased GAG content,¹⁴² as one of the roles in these zones is to provide resistance against compressive stresses.

The feasibility of developing stratified cartilage is supported by the fact that three-dimensional culture of chondrocytes from different zones maintain the specific metabolic activities of the zonal population,^{142,145} and preliminary efforts to regenerate zonal organization in articular cartilage tissue have been attempted. In one instance, constructs were seeded with ARC cultured chondrocytes from the superficial zone on top of ARC cultured chondrocytes from the middle zone chondrocytes.¹⁴⁶ In the second study, zonal chondrocytes were incorporated into separate layers using a photopolymerizing hydrogel.²⁹ No cross-contamination between the layers was observed upon construct harvest in either study, and the tissues exhibited similarity to native cartilage tissue including gradients of proteoglycan accumulation or secretion of superficial zone protein

mainly from the superficial zone chondrocytes. Given these initial attempts, it is possible to find a role for PLG microspheres in stratified cartilage regeneration, most likely in a composite scaffold. As cells readily attach to microspheres, specific zonal populations of chondrocytes could be incubated with microspheres during the 4 hour attachment period. Moreover, the encapsulated contents in the microspheres could be tailored to induce desired effects of the specific population of chondrocytes. PLG microspheres with attached chondrocytes could be layered in a mold or could be further encapsulated in another polymer, such as hydrogels like alginate or poly (ethylene oxide).

As a pre-requisite to clinical trials for any tissue engineering model, the feasibility for cartilage repair must be demonstrated in a large animal model. Typically, initial studies involve proof of principle using in vitro culture or in vivo, subcutaneous evaluation.¹⁴⁷ Subcutaneous evaluation has most often been demonstrated in small animals such as mice or rats,^{28,37} but larger animals such as the ovine model have also been used.^{28,37} Subcutaneous culture of cartilage constructs evaluates the growth capabilities of the tissue and allows for initial determination of tissue properties such as matrix accumulation, but does not assess the ability of the method to repair a defect. Some studies, however, have determined the ability of specific cartilage regeneration methods to repair defects in large animal models, most notably in canine or equine.^{68,141} Repair in these defects are not only judged on similarities between matrix content and mechanical properties of the engineered tissue and native cartilage tissue, but also the importance of engineered tissue integration with endogenous tissue must be reconciled.

The current study evaluated a novel cartilage tissue engineering method using a simultaneous scaffold and growth factor delivery vehicle, demonstrating proof of principle and identifying specific polymer and scaffold characteristics that can lead to enhanced cartilage repair. A consequent follow-up to this study would include the evaluation of this cartilage tissue engineering method in a large animal model, with specific attention to tissue repair, including integration of the engineered/endogenous cartilage interface, evaluation of matrix synthesis and production, and determination of mechanical and weight bearing properties.

Significance

In a field such as tissue engineering, understanding basic interactions in the tissue engineering design will build a foundation for subsequent studies. There are multiple facets to the significance of this work including understanding the contribution of polymer and scaffold to cartilage growth and developing a novel, growth factor delivery scaffold for cartilage tissue engineering. Both of these advances suggest methods to enhance cartilage tissue development and may have potential for cartilage repair.

A first novelty of the work presented in this thesis demonstrates initial attempts at understanding the effects of the polymer scaffold on tissue regeneration. To date, there has been relatively little work that has involved an understanding of pointed changes in the polymer or scaffold and the effect on tissue formation. However, preliminary research has suggested that polymer type and pH can affect cartilage tissue engineering.^{50,79,155} Specifically, fewer chondrocytes attached to PLA versus PGA,¹⁵⁵ and increasing the ratio

of PLA in a PLA/PGA co-polymer decreases chondrocyte attachment.⁷⁹ Similarly, drops in pH led to inhibition of matrix assembly.⁵⁰ In contrast, the work provided in this thesis marks the first broad range analysis of multiple polymer and scaffold properties that influence cartilage tissue growth. Most notably, using increased molecular weight microspheres in tissue formation generates cartilage with increased ECM and tissue mass, while release of $\text{Mg}(\text{OH})_2$ inhibited matrix and mass accumulation (Chapters II and III).

Although we have evaluated polymer and scaffold properties and the effects on tissue formation, we have only evaluated parameters for PLG. As there are multiple types of scaffolds used for cartilage tissue engineering, it will be important to understand which properties will enhance cartilage formation in other polymers, such as agarose, alginate, fibrin, collagen, PGA, PLA, chitosan or other polymers. In addition, this study assessed a limited number of variables that affect tissue formation. It would be important to further investigate how molecular weight enhances tissue formation (Chapters II and III) by evaluating a range of polymer molecular weights and assessing a variety of parameters including integrin expression and cell adhesion. Similarly, it is important to assess potential reasons for magnesium hydroxide inhibition of matrix and tissue growth (Chapters II and III); specifically, important avenues of exploration would include analyzing a range of buffer loads that provides an effective buffer without cellular toxicity or assessing various, physiological buffers such as HEPES or PBS.

A second novelty arising from this work focused on a scaffold with criteria to provide for cell attachment and tissue support, as well as growth factor delivery. The importance of this system is non-trivial and can be understood from the drawbacks of

current techniques. First, a dichotomy exists in scaffold properties and invasiveness of the repair procedure. PLG type polymer chains can be easily regulated by controlling factors that affect degradation rate include capping the polymer, adding a buffer to the microsphere, or changing properties like molecular weight, microsphere size, porosity, or pore size (ref. 23, Chapter II and III). Additionally, these polymers are often shaped and require surgical implantation. In contrast, polymers such as alginate or collagen have fewer factors that can be changed to influence scaffold properties, but the injectability of these materials make them advantageous for non-invasive procedures.

In our work, we have chosen to utilize PLG microspheres, which offer both an ease of manipulation of polymer properties and minimization of invasiveness due to the small, spherical nature of PLG microspheres. Chondrocytes and microspheres can be injected *in vivo* or molded *in vitro* (Chapters II-V). Moreover, chondrocytes attach to PLG microspheres (Appendix A), PLG microspheres support tissue growth (Chapters II-V), and PLG microsphere properties can be changed to influence tissue formation (Chapters II-V).

Another facet overcome by PLG microspheres is the limitation of growth factor half-life when added by exogenous delivery. The ProLease fabrication process of PLG microspheres with encapsulated protein has demonstrated almost 99% retention of protein stability and activity,⁸⁹ and in this study we have demonstrated effective release of IGF-I out to 8 weeks (Chapter IV) with around 70% total release of IGF-I after 8 weeks (Chapter V). Moreover, we have demonstrated that controlled release of IGF-I in

combination with high molecular weight microspheres can be used to further enhance cartilage formation (Chapters IV and V).

Although not determined in this study, it is likely that PLG microspheres can be used to deliver multiple growth factors. It has been shown that growth factors, such as TGF- β and IGF-I, have synergistic effects on tissue formation.²⁵ Because the polymer properties of PLG microspheres can be easily changed to affect degradation rate, it is likely that our system could utilize two sets of PLG microspheres with the same degradation times to release growth factors at the same rate for synergistic actions, or by using microspheres with different degradation times, one growth factor could be released prior to a second. Further, it would be important to understand how changes in polymer and scaffold parameters affected protein release, which was not in the scope of this study.

Similar synergy has been demonstrated with mechanical stimulation and exogenous IGF-I addition.^{168,169} Although not the focus of this study, it would be important to understand synergistic effects of mechanical stimulation, such as compression or fluid flow, with controlled release of IGF-I to make specific comparison to the synergy of exogenous delivered IGF-I and mechanical stimulation.

In general, our study has provided specific and novel contributions to tissue engineering. We have developed a novel polymer scaffold with simultaneous growth factor release that overcomes the limitations of invasive procedures and protein half-life, two important factors in the tissue engineering field. Moreover, we have demonstrated that the polymer scaffold does influence tissue regeneration. Although there are limitations to our studies, namely a more thorough investigation of ranges in specific

polymer properties (i.e. a range of molecular weight) that could further enhance of tissue growth, the level of regulation (gene, protein, or matrix assembly) at which the polymer influences tissue regeneration, and control of this system to incorporate synergistic factors (additional growth factors or mechanical stimulation), this work provides a foundation for future directions to understand the critical role of the polymer scaffold in cartilage tissue engineering.

APPENDIX A

CARTILAGE ENGINEERED WITH PLG MICROSPHERES EXHIBIT

SIMILARITIES TO NATIVE CARTILAGE: A LOOK AT CELL

ATTACHMENT AND SPACING

Introduction

Cartilage tissue is uniquely structured with a high matrix to cell ratio, and thus the low cell density, less than 10% of the overall tissue volume, does not promote the formation of cell-to-cell contacts² typically seen in other tissues. In contrast, chondrocytes attach to and are thus embedded in a dense network of ECM molecules, for example, collagen type II, fibronectin, and hyaluronan. The attachment of chondrocytes to ECM molecules enables cross-talk and signaling within the tissue, which respond to stimuli such as growth factors and mechanical loading.⁸ Thus, the issues of cell spacing and chondrocyte attachment in engineered cartilage must be of primary focus.

The importance of cellular attachment to the synthetic polymers has been well established,^{148,149} and variations in polymers can affect cell attachment. For example, polymer properties such as hydrophobicity/hydrophilicity can impact cell adhesion, as hydrophilic polymers enhance cell attachment, while hydrophobic polymers decrease cell attachment.^{79,150,151} In addition, manufactured attachment motifs such as the peptide RGD can facilitate attachment.^{152,153} Alginate is a polymer that does not inherently facilitate cell adhesion, as this polysaccharide does not provide attachment for

mammalian cells and its negative charge electrostatically repels protein adsorption.¹⁵² However, modifying alginate to include the RGD peptide enables cells to attach and spread in alginate hydrogels.¹⁵⁵

In contrast, attachment of chondrocytes to unmodified PLG scaffolds has been demonstrated without the need for such polymeric modifications and adhesion has been demonstrated for PLG scaffolds of varying shapes, including fibers and films.^{20,79,155} Although adhesion of chondrocytes to PLG microspheres has not been demonstrated, the feasibility of chondrocyte attachment to minute shapes has been demonstrated for other polymer types, including microparticles composed of collagen type I or dextran.⁵²

Because chondrocytes have been shown to attach to both PLG type polymers and minute sized non-PLG polymers, such as microparticles, this suggests that chondrocytes will attach to PLG microspheres. Thus, the goal of the studies presented in this appendix was to determine whether chondrocytes attached to PLG microspheres, and subsequently to determine the cell spacing in cartilage tissue engineered with only chondrocytes or with chondrocytes and PLG microspheres.

Materials and Methods

Cell Culture

Chondrocytes were isolated from the articular cartilage of calf gleno-humeral joints of 2-10 day old calves (Research 87, Hopkinton, MA) using 0.3% type II collagenase (Worthington), as previously described.⁷⁶ After 16 hr of collagenase treatment at 37°C, isolated chondrocytes were filtered through a 180 μ m filter to remove any undigested

cartilage particles, washed several times with PBS, and cells were resuspended in Ham's F12 media supplemented with 1% ascorbic acid and 10% FBS.

Microsphere Preparation

PLG was obtained from Alkermes, Inc. PLG microspheres were fabricated using the ProLease[®] process. Briefly, PLG polymer (50:50 lactide:glycolide, uncapped (-COOH), $M_w \sim 10$ kDa) was dissolved in methylene chloride and atomized over a bed of liquid nitrogen layered on top of frozen ethanol. The temperature of the system was then maintained at -80°C to allow for nitrogen evaporation and removal of methylene chloride into the ethanol phase (microsphere curing). The resulting microspheres were filtered and then lyophilized to remove residual ethanol, and finally sieved to obtain microspheres with an average diameter of $35\ \mu\text{m}$.

Attachment Kinetics

PLG microspheres were suspended at 3.6 mg microsphere/ml of 0.3% carboxymethyl cellulose (Sigma Chemical Company, St. Louis, MO) in F-12 media and mixed with 1×10^6 chondrocytes/ml at 37°C . Samples (1 ml) were removed at 0, 2, 4, and 16 hr and separated on a Histopaque-1077 density gradient (Sigma Chemical Company) by centrifugation for 5 min at 5000 rpm. DNA content of the top (unattached) and bottom (attached) fractions were assayed using the Hoechst 33285 Dye Method, as described (Kim 1988). Briefly, Hoechst 33258 dye (Sigma Chemical Company) was diluted 1:10000 in TEN buffer (Tris-EDTA-Sodium Chloride) to make the dye stock, and 2 ml of

Hoechst dye stock was added to 50 μ l of each gradient fraction. Fluorescence was read at 358/458 nm. Calf thymus genomic DNA (Sigma Chemical Company) was used as a standard.

Culture Conditions and Implantation

Chondrocytes, at a density of 80×10^6 cells/mL, were mixed with an equal volume of PLG microspheres, at a concentration of 64 mg/mL. The microsphere-chondrocyte suspension was incubated for 4 hr at 37°C in a shaker incubator to allow for chondrocyte attachment to the microspheres.⁸²

Chondrocyte, microsphere, and chondrocyte-microsphere suspensions were injected into the subcutaneous space of the dorsum of nude mice using an 18-gauge needle. Each injection contained 20×10^6 cells and/or 16 mg of microspheres in 500 μ L of F12 media.

Sample Analysis

All animal procedures were performed under the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. Mice were sacrificed at 1, 2, 4, 6, and 8 weeks by anesthesia overdose and samples were harvested from cell alone and chondrocyte-microsphere samples. No tissue samples could be harvested from microsphere alone injections, as there was no tissue formation at the site of injection.

Upon harvest, the fibrous capsule was removed from each sample, and the tissue was either processed for bulk frozen fracture scanning electron microscopy, as described

below, or fixed in 10% formalin, embedded in paraffin, and sectioned. Sections were stained with safranin-O.

Scanning Electron Microscopy

PLG microspheres were suspended at 3.6 mg microsphere/ml of 0.3% carboxymethyl cellulose in F-12 media and mixed with 1×10^6 chondrocytes/ml at 37 °C. Samples (1 ml) were removed at 0, 2, 4, and 16 hr and were pipetted onto SEM slides covered with Tissue Tack (Sigma Chemical Company). Samples were fixed in 2.5% glutaraldehyde overnight at 4°C, and subsequently dehydrated in a graded series of ethanol concentrations up to 95% ethanol. Slides were sputter coated with approximately 10 nm Au/Pd and examined on an ETEC Autoscan Scanning Electron Microscope (Perkin Elmer, Wellesley, MA) at 20 Kv.

Alternatively, cartilage tissue engineered from chondrocytes alone or chondrocytes and microspheres was harvested after 1 week of in vivo subcutaneous incubation and was subjected to bulk frozen fracture according to a protocol by Hendricks and Hadley.¹⁵⁷ As described, tissue samples were fixed in 2.5% glutaraldehyde overnight at 4°C, and subsequently dehydrated in a graded series of ethanol concentrations up to 95% ethanol. The partially dehydrated tissue was then quickly immersed in liquid nitrogen until the boiling ceased, placed on a pre-cooled steel block, and fractured using a cooled knife. The fractured pieces were collected, thawed in 100% ethanol, and critical point dried in a CO₂ CPD system. Once dried, the pieces were mounted on aluminum stubs with silver conductive paste and sputter coated with

approximately 10 nm Au/Pd and examined on an ETEC Autoscan Scanning Electron Microscope at 20 Kv.

Histomorphometry

The internuclear distance between cells ($n=10,000$) was measured using Scion Image Software (Scion Corporation, Frederick, MD) (Figure 30a) in cartilage engineered from only chondrocytes and from cells and microspheres at 2, 4, or 8 weeks, as well as native cartilage tissue. Distances were averaged, values were pooled to form a histogram chart, and evaluated for each time point (Figure 30b).

Results

Chondrocytes bound to PLG microspheres as evidenced by SEM (data not shown) and biochemical analysis (Figure 31). Chondrocyte attachment was observed by SEM at 2 hours (not shown), and a time constant (τ) of approximately 5 hours was calculated from the attachment kinetic curve (Figure 31). Chondrocytes exhibited first order binding kinetics with PLG microspheres with a maximum of 30% attachment to microspheres (Figure 31).

Chondrocyte attachment within engineered cartilage was also assessed using SEM. After 1 week, bulk frozen fracture samples from cartilage formed with chondrocytes and microspheres indicated a microsphere network that was connected by chondrocytes and matrix tissue (Figure 32A). Chondrocytes appeared to contour the curvature of the microsphere but retained the normal spherical phenotype (Figure 32B),

and evidence of attachment was indicated by processes projecting from the chondrocyte that contacted the surface of the microsphere (Figure 32C). Cartilage formed with chondrocytes and microspheres generated tissue that maintained its structural integrity and did not show evidence of fibrous tissue. In contrast, cartilage formed using cells alone indicated cracks in the tissue that separated the cartilage layer from more fibrous tissue (Figure 32D).

The internuclear distance of chondrocytes was measured in tissue generated from chondrocytes and microspheres and chondrocytes alone. At weeks 2 and 4, cells spacing was compact in all samples analyzed, and internuclear distances were similar for cartilage formed from cells and microspheres and that formed from only chondrocytes. After 8 weeks, the internuclear distance between chondrocytes increased in all samples, but cartilage engineered from cells and microspheres exhibited a significantly higher spacing of 28 μm as compared to a 24 μm spacing seen in cartilage formed from only chondrocytes ($p < 0.0001$) (Figure 33A).

Total cell densities could be calculated from spatial distances in these samples. When analyzed as such, cell densities at weeks 2 and 4 indicated little variation between the groups at cell densities of $80\text{--}85 \times 10^6$ cells/ml. However, after 8 weeks, cell densities were decreased in all samples, but cartilage formed with cells and microspheres had significantly lower cell densities (45×10^6 cells/ml) than cartilage formed with chondrocytes alone (75×10^6 cells/ml). Moreover, cartilage generated from cells and microspheres exhibited cell numbers that were more similar to native cartilage values (Figure 33B).

Discussion

As the goal of cartilage tissue engineering is to form tissue with close similarities to native cartilage tissue, cell attachment to the polymer scaffold and subsequent and appropriate cell spacing is necessary. These adhesion parameters have been studied for scaffolds made from PLG in various forms,^{20,79,155} but these characteristics have not been determined for PLG microspheres. Thus, this study documented both the attachment kinetics of bovine articular chondrocytes to PLG microspheres and the changes in cellular spacing with time in engineered cartilage tissue.

Cell attachment to the polymer scaffold is vital to engineering of new tissue,¹⁴⁸ and consequently, the first goal in this study was to determine the characteristics of adhesion to PLG microspheres. SEM indicated chondrocyte attachment as early as 2 weeks, and kinetics demonstrated chondrocyte attachment to PLG microspheres increased over the first 12 hours, with an adhesion time constant of 4-5 hours. In addition, the total amount of chondrocytes that adhered to PLG microspheres was 30% of the total seeded chondrocytes. The percentage of cells adhering to PLG microspheres was consistent with other attachment studies that indicated chondrocyte attachment to PLA/PGA composite scaffolds at 27%.⁷⁹ In addition to adhesion kinetics, the appearance of cells has also been used as an attachment indicator, characterized by cell spreading and protrusion of processes that attach to substrates.^{152,154} In this study, chondrocytes clearly maintained their rounded morphology in cartilage formed with chondrocytes alone (data

not shown) or chondrocytes and microspheres (Figure 32B), yet cellular processes that contacted microspheres were observed in cartilage from cells and microspheres.

Chondrocyte and tissue appearance also gave indications of the overall tissue integrity. Cartilage tissue from chondrocytes alone indicated evidence of cavities that were lined with fibrous tissue, but these cavities were not exhibited in cartilage formed with cells and microspheres. Since the polymer scaffold is known to provide strength and support to the growing tissue and the chondrocyte protrusions contacted and appeared to attach to PLG microspheres, it is likely that PLG microspheres provide a solid support scaffold for cartilage tissue engineering. Moreover, without this scaffold support, the structural integrity of the tissue was compromised, which has been shown to have severe implications in intrinsic cartilage repair, as production of fibrocartilage generates tissue with inferior biomechanics, tissue breakdown, and osteoarthritis.¹⁵⁸⁻¹⁶⁰

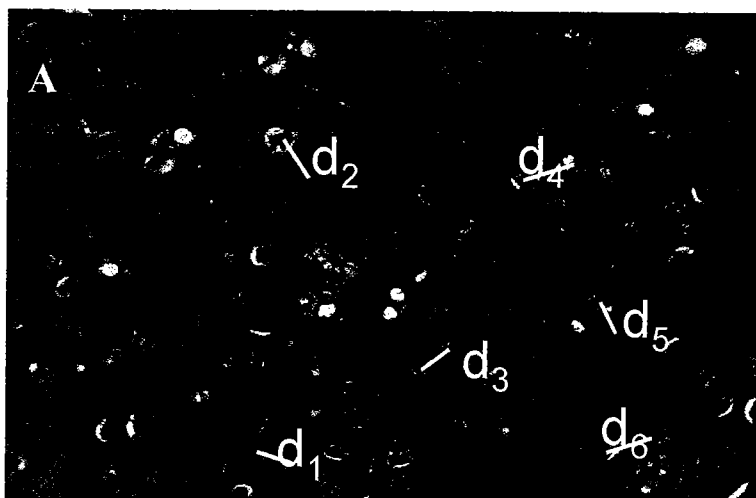
Another important characteristic that the scaffold must lend to the developing tissue is room to expand. In many tissue engineering designs, matrix deposition has correlated with polymer degradation,²² and often the scaffold provides a predetermined 3-dimensional shape and volume for tissue growth and matrix deposition.^{20,26} In contrast, a predetermined 3-dimensional volume is not available for matrix deposition when PLG microspheres are used as a scaffold; rather, tissue volumes exhibited in this study were attributed to increases in ECM deposition only. Thus, in tissue engineering applications with PLG microspheres, microspheres likely act to maintain additional space for cell migration and matrix deposition as the tissue forms.

This theory was evidenced by changes in cell spacing and cell densities as cartilage formed with or without PLG microspheres developed over 8 weeks. From histological analysis presented in chapter II and III, it appears that chondrocytes are confined to areas outside of the microspheres until the microspheres begin to degrade. Once degradation ensues, chondrocytes may migrate into the degrading microsphere and deposit matrix. In this study evidence of cell spacing in chondrocyte and microsphere samples coincided with degradation patterns of the microspheres (data not shown and ref. 23), and thus at time points of 2 to 4 weeks, when microspheres have not yet degraded, internuclear distances in cartilage tissue from both sample sets did not differ. Yet, after 8 weeks, when degradation in microspheres has occurred, significant variations in internuclear distances were detected; chondrocytes were more spaced in cartilage formed using chondrocytes and microspheres than cartilage formed from chondrocytes alone. Similarly, differences in cell density between the groups were not apparent after 2 and 4 weeks, but significant differences between the groups after 8 weeks. Over the time of the study, cell density dropped slightly in cartilage formed with chondrocytes alone, but maintained a much higher density than that of native cartilage tissue. In contrast, the cell density in samples formed with cells and PLG microspheres exhibited cell densities consistent with those of native cartilage tissue. Other studies have attempted to engineer cartilage tissue by culturing chondrocytes as pellets or in alginate, and while evidence exists for maintenance of chondrocyte phenotypic markers in these systems, hypercellularity and lack of cell spacing remain problematic.^{16,17,19} These data and the data presented in this study suggest that tissue formation with only chondrocytes limits

the cell spacing and cell density by not providing any additional space for cell migration. However, in cartilage cultured with PLG microspheres, the additional space leant by the PLG microsphere scaffold enables appropriate cell spacing and cell density.

In native cartilage tissue, cells are widely spaced so as not to form cell-cell contact. Individual chondrocytes are immediately surrounded by a pericellular matrix, which is high in proteoglycans and type VI collagen. Matrix further from the cell consists of a territorial and an interterritorial matrix, the latter forming the majority of the matrix with collagen type II and aggrecan.² A lack of appropriate cells spacing may shift the balance of matrix constituent concentrations, which in turn would change the properties of the tissue.

Overall, this study has demonstrated that chondrocytes actively attached to PLG microspheres over the first 12 hours of incubation. Moreover, as PLG microsphere degradation proceeded, spacing of chondrocytes in the engineered cartilage tissue increased and exhibited cell densities on the level of native cartilage tissue. Taken together, these data suggest that PLG microspheres can support cartilage tissue engineering and maintain space for additional tissue growth.



$(d_1, d_2, d_3, \dots d_n)$



B

Cell Spacing

$n = 10,000$ cells

Mean = 23.2 μm

StDev = 7.6 μm

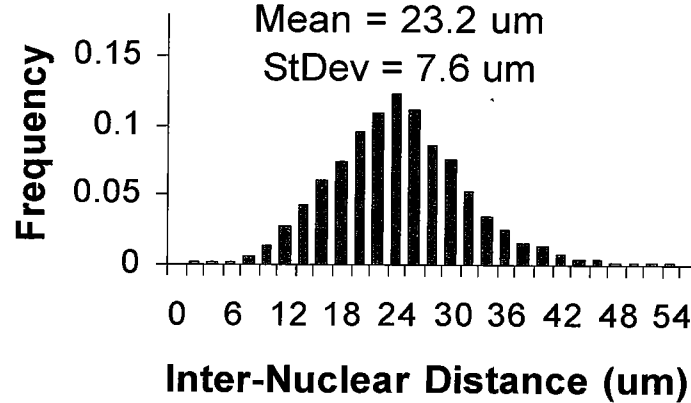


Figure 30. Panel A depicts a safranin-O stained cartilage section. Distances between nuclei were calculated using NIH Scion Image. After all nuclei distances were calculated, distances were scored for frequency and charted in histograms as depicted in Panel B.

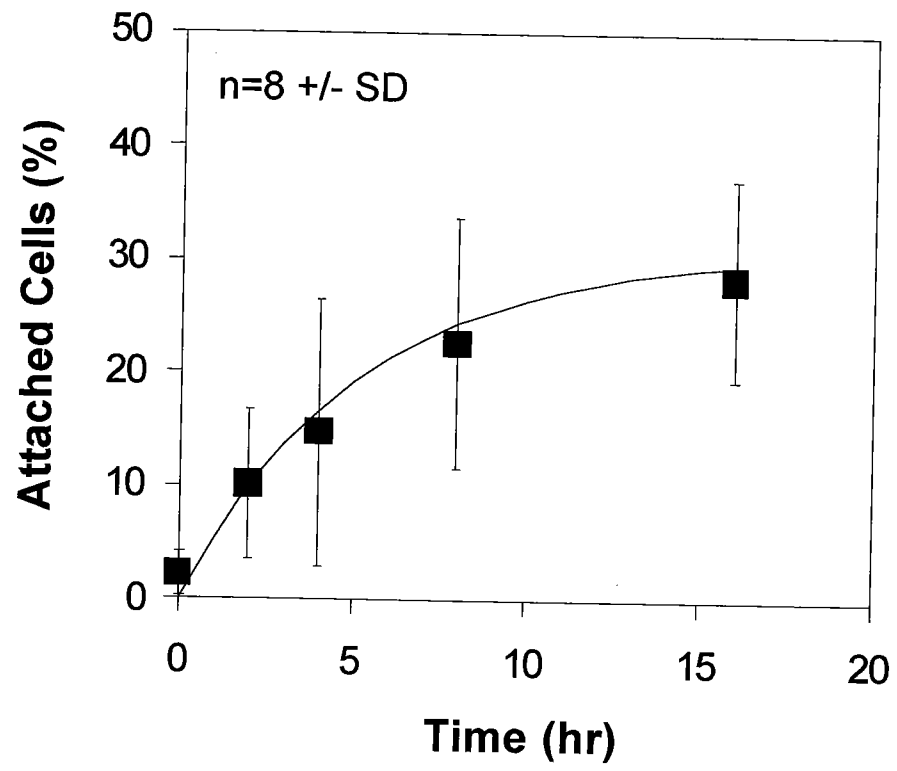


Figure 31. Chondrocytes attached to PLG microspheres. Chondrocytes and microspheres were incubated over 16 hr. At each time point, unattached cells were separated from microspheres and attached cells using a density gradient. Both attached cell samples and unattached cell samples were detected by fluorescence, as described in materials and methods. Attachment of chondrocytes was exhibited as early as 2 hr and increased linearly until 8 hr. After 16 hr, about 30% of cells in the initial cell load had attached to microspheres.

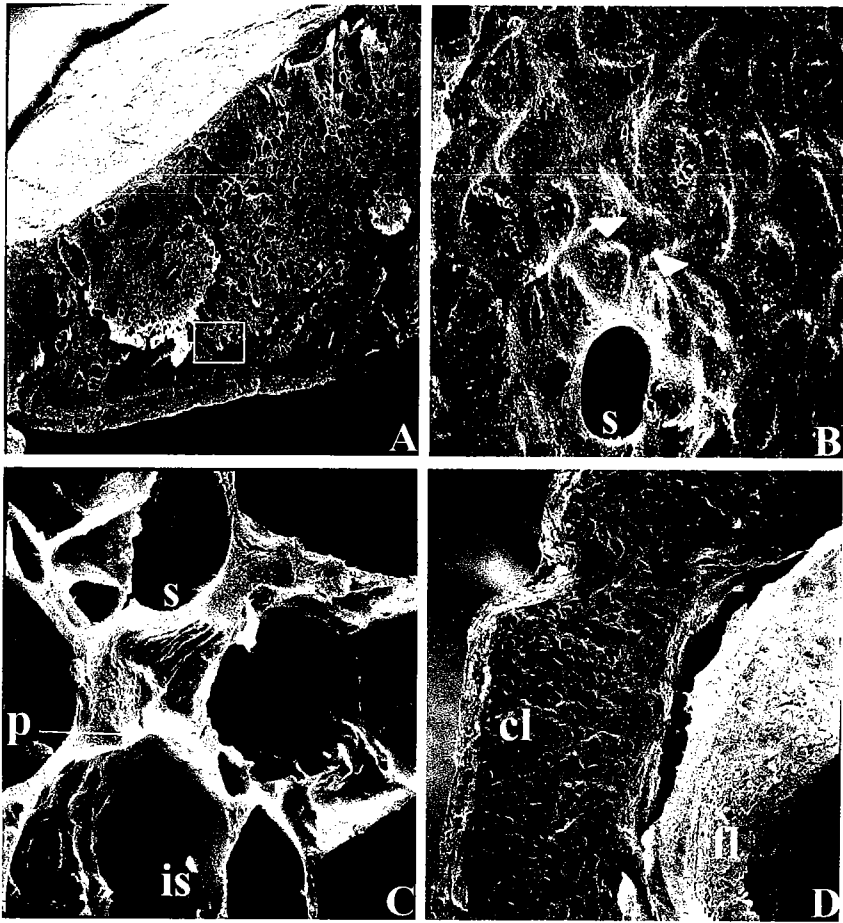
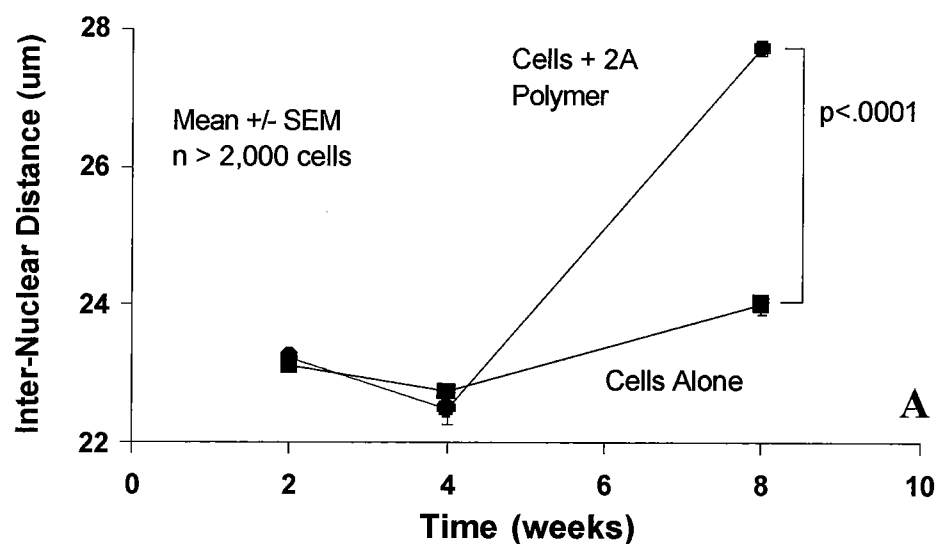


Figure 32. Chondrocyte attachment to PLG microspheres can be observed via scanning electron microscopy. Cartilage formed with cells and microspheres (A-C) or cartilage formed from cells alone (D) was subjected to bulk frozen fracture and magnified at 50x (A), 1000x (B), 2000x (C) and 200x (D). Chondrocytes appeared to contour microspheres (B), indicated by yellow arrowheads, and form attachments via cellular processes (C), indicated by (p). The fracture process produced both empty pockets thought to have been occupied by microspheres (s) and intact microspheres (is). Cartilage formed from cells alone exhibited a layer that appeared to have rounded chondrocytes (cl) and a fibrous-like layer in the center of the tissue (fl).

Chondrocyte Spacing in Engineered Tissue



Cell Density in Engineered Tissue

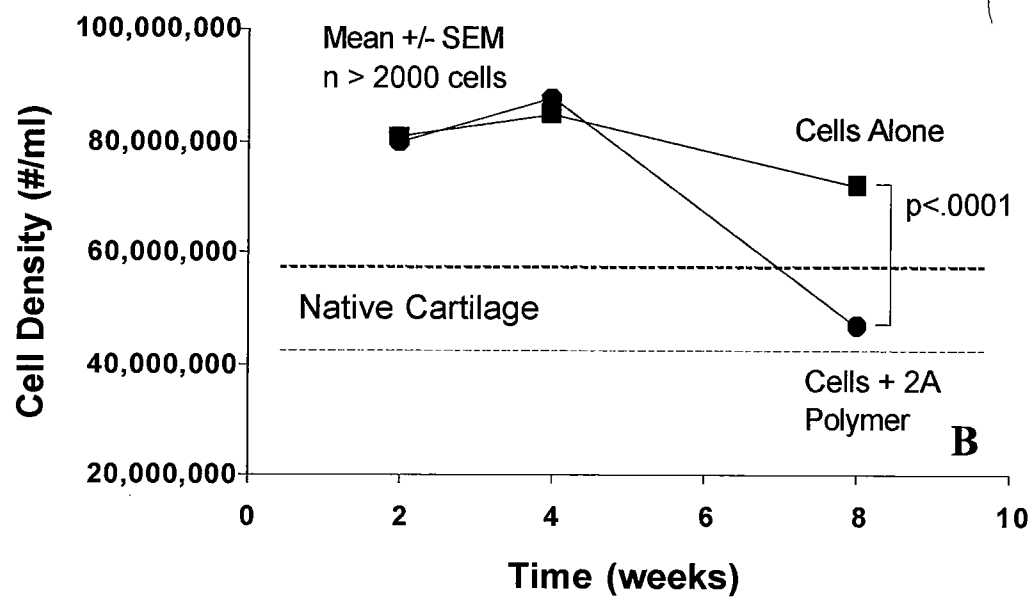


Figure 33. A. Internuclear distances in cartilage engineered from cells alone or cells and microspheres were assessed over 8 weeks. No differences in cell spacing were evident at 2 or 4 weeks, but after 8 weeks chondrocytes in cartilage formed from cells and microspheres had significantly higher internuclear distances than in cartilage formed from cells alone (A). Similarly, cell density between the two groups was not different at 2 or 4 weeks, but after 8 weeks, the cell density in cartilage formed with chondrocytes and microspheres dropped to native cartilage levels. In contrast, cartilage formed from cells alone exhibited high cell density after 8 weeks.

APPENDIX B

GENE TRANSFER OF IGF-I MAY PROVIDE AN ALTERNATIVE DELIVERY METHOD FOR USE IN CARTILAGE TISSUE ENGINEERING

Introduction

The utility of growth factors in tissue engineering has been recognized as advantageous, and many studies have exploited the use of these growth factors to maintain cell differentiation or enhance matrix production. Typically, growth factor is exogenously added to the tissue engineering construct, but is limited by protein half-life⁹⁰ and must be added with each media change.^{24,32} However, novel alternatives to exogenous delivery are actively being sought, and gene transfer has recently been evaluated for use in tissue engineering studies.

Cells can be genetically modified to overexpress growth factors by a multitude of methods. In cartilage tissue engineering genetic modification has mostly been attempted by transfecting chondrocytes. Chondrocytes transfected with a plasmid containing the IGF-I gene stimulated a new layer of tissue on cartilage explants⁷⁰ and formed cartilaginous tissue when seeded in PGA scaffolds.⁶⁹ Similarly, transduction of chondrocytes with adenoviral vectors can effectively be used for gene transfer, and the

feasibility of transducing chondrocytes with an adenoviral vector containing the IGF-I gene has been demonstrated.¹⁶¹ However, recombinant adenovirus with IGF-I has not been used for tissue engineering applications.

It was the goal of this study to generate an adenoviral construct containing the IGF-I gene for use in cartilage tissue engineering.

Materials and Methods

Cloning of IGF-I Gene into Adenoviral Vector

The pT7blue3-hIGFI plasmid containing the IGF-I cDNA, a gift from Dr. Dan Grande, was digested with ClaI overnight at 37°C, followed by HindIII (New England Biolabs) for 3 hr at 37°C to remove the IGF-I gene from the vector. Overhanging ends were filled using Klenow (New England Biolabs, Beverly, MA) for 1 hr at 37°C. pShUiT, a gift from Dr. Madelyn Schmidt, was linearized with EcoRV (New England Biolabs) and 5' phosphates were removed with calf intestinal phosphatase (New England Biolabs). The filled IGF-I fragment was ligated into the linearized pShUiT in the presence of polyethylene glycol 6000 (Fisher Scientific, Hampton, NH) with T4 DNA ligase (New England Biolabs). Potential clones containing the IGF-I gene were screened using polymerase chain reaction (PCR) with primers IGF forward 5'-CCCAAGCTTATGGGAAAAATCAGCAGTCTTCCAACCCAA-3' and IGF reverse 5'-CGCGGATCCCTACATCCTCTAGTTTCCTGCACT-3'.

Transfection of Human Kidney Epithelial Cells with IGF-I

Human kidney epithelial (293) cells were transfected with 0, 1, or 10 μ g of pShUiT-IGFI according to the protocol for Lipofectamine Reagent. Briefly, pShUiT-IGFI DNA was mixed with Opti-MEM I (Gibco, Carlsbad, CA) and Plus Reagent (Invitrogen Life Technologies, Carlsbad, CA) and incubated at room temperature for 15 min. Lipofectamine Reagent (Invitrogen Life Technologies) was diluted in Opti-MEM I and 50 μ l diluted lipofectamine was added to each tube of DNA. DNA and lipofectamine were incubated together for 15 min at room temperature and then transferred to 293 cells (80% confluent) in a 6 well plate. DNA-lipofectamine was allowed to incubate for 3 hr at 37°C and then F-12/D-MEM media was added to each well. Cells were allowed to recover for 72 hr, after which media was removed for IGF-I detection by ELISA.

IGF-I ELISA

Media was assayed for IGF-I content using the Human IGF-I Quantikine ELISA kit according to the manufacturers specifications (R&D Systems, Inc, Minneapolis, MN). IGF-I standards and collected media were incubated on an ELISA plate pre-coated with mouse monoclonal IGF-I antibody for 2 hr at 4°C. Unbound samples were washed from plate and the plate was incubated with an IGF-I polyclonal antibody conjugated to horseradish peroxidase at 4°C. Excess secondary antibody was washed from the plate and wells were incubated with hydrogen peroxide/chromogen for detection of IGF-I. Optical densities were read at 450nm.

Results

Four clones containing the IGF-I insert were identified by PCR with primers specific for the 5' and 3' termini of the IGF-I ORF (Figure 34). Repeated attempts to determine orientation of the IGF-I ORF by restriction digestion were unsuccessful. Therefore, each construct was transfected into human kidney epithelial cells. Screening of supernatants of the transfected cells for the presence of IGF-I at 72 hr post transfection by ELISA were also unsuccessful.

Discussion

In this study we successfully cloned the IGF-I gene into the pShUiT adenoviral vector, however, we did not see IGF-I expression in transfected 293 cells. One possibility for lack of IGF-I expression is that the gene was ligated into pShUiT in reverse orientation to the vector derived ubiquitin promoter. Similarly, poor transfection of 293 cells with IGF-I DNA could explain the lack of IGF-I expression. Future studies would need to re-transfect 293 cells or another suitable cell line to obtain efficient transfection conditions.

The importance of this study provides preliminary techniques for generating an adenoviral vector that contains the IGF-I gene. Transduction of chondrocytes with adenoviral vectors could provide an alternative delivery method for adding growth factor to the tissue engineered construct, and the utility of this method would be advantageous

because it offers the potential for genetic transduction in vitro¹⁶¹ or in vivo gene transfer via direct injection.¹⁶²

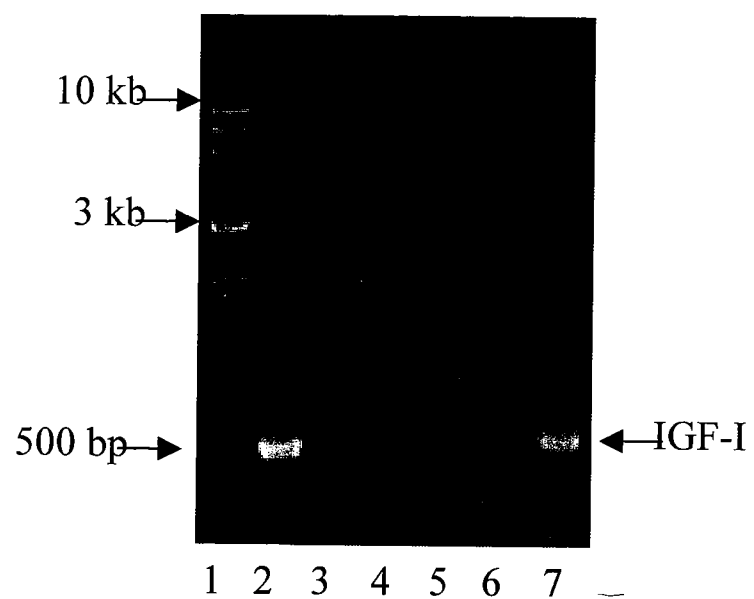


Figure 33. pShUiT-IGFI contains the IGF-I gene. The IGF-I ORF was amplified by PCR from purified plasmid DNA recovered from candidate clones. PCR products were analyzed by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining. Lane 1. 1 kb Ladder (New England Biolabs); Lane 2. pT7blue3-hIGFI (control template); Lanes 3-7. pShUiT-IGFI candidate clones.

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